NOTE TO USERS

Mechanisms of TrkA-induced cell death of neuroblastoma

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Abstract:

Neuroblastoma is the most frequent solid extra-cranial tumor in children. Its clinical prognosis correlates with the expression pattern of members of the Trk neurotrophin receptor family, which includes TrkA, TrkB, and TrkC. TrkA expression is associated with favorable prognosis, while TrkB expression is associated with poor prognosis. Here we show that TrkA overexpression also induces neuroblastoma apoptotic cell death, and does so by modulating the levels or activities of a number of proteins involved in regulating cell survival and apoptosis, including p53, Bcl-2 and caspase-3. In addition, treatment with a caspase inhibitor or overexpression of Bcl-X_L prevented TrkA from inducing apoptosis. These results identify apoptosis as a novel biological response of TrkA, implying that TrkA is a good prognosis marker for neuroblastoma due to its ability to induce apoptosis when highly expressed, and suggest that therapies based upon TrkA overexpression or stimulation will be efficacious for the treatment of neuroblastoma.

Résumé:

Le neuroblastome est la tumeur extra-crânienne solide la plus fréquente chez les jeunes enfants. Son pronostique est associé à l'expression de membres des récepteurs neurotrophiques, incluant, TrkA, TrkB et TrkC. L'expression de TrkA est associée à un bon pronostic tandis que l'expression de TrkB est défavorable. La présente étude démontre que la sur-expression de TrkA induit la mort par apoptose des neuroblastomes en modulant l'expression ou l'activité de protéines, tel que p53, Bcl-2 et caspase-3, qui sont impliquées dans la régulation de la survie cellulaire et de l'apoptose. De plus, la présence d'un inhibiteur des caspases, ainsi que la sur-expression de Bcl-X_L, empêche TrkA d'induire l'apoptose. Cette découverte, que la sur-expression de TrkA induit l'apotose de neuroblastome, confirme que TrkA est un marker de bon pronostic et prédit l'efficacité d'une thérapie basée sur la sur-expression de TrkA, ou sur sa stimulation, dans le traitement des neuroblastomes.

Chapter I: Regulation of cell death in neuroblastoma: Role of neurotrophin receptors

1.1 Introduction

Neuroblastoma, the most common solid extracranial tumor of childhood, is a biologically fascinating, but clinically deadly pediatric tumor. The median age at diagnosis is approximately 18 months and spontaneous tumor regression is frequently observed in patients diagnosed at one year of age or younger. In contrast, children older than one year diagnosed with neuroblastoma often experience aggressive tumors that are disseminated, resistant to chemotherapy, metastasized to bone and often fatal. One correlative characteristic of neuroblastoma is the expression of the neurotrophin tyrosine kinase receptors, TrkA and TrkB. The expression of TrkB, a poor prognosis marker, mediates survival, proliferation, and chemotherapeutic drug resistance, while expression of TrkA, a favorable prognosis marker, induces cell growth arrest and differentiation of cultured neuroblastoma cells. In addition, preliminary findings from our laboratory suggest that TrkA expression induces the death of neuroblastoma cells. My Master's thesis focuses on confirming this result and on determining the molecular mechanism by which TrkA induces cell death. As a general introduction to my Master's thesis data, this chapter addresses the following questions. What is the origin of neuroblastoma? What are the cell death pathways identified in neuroblastoma? How do correlative prognosis markers such as TrkA mediate their biological effects and what are the Trk signal transduction pathways in neuroblastoma cells? Answers to these questions will lead to the development of hypotheses on how TrkA induces the death of these cells.

1.2 General background on neuroblastoma tumors

The etiology of neuroblastoma remains unknown, but all available evidence supports the hypothesis that they arise from sympathoadrenal precursor cells. This is supported by three observations. Firstly, the site where neuroblastomas occur is the same as where embryonic sympathoadrenal progenitor cells migrate to give rise to the sympathetic nervous system and adrenal medulla (Bohn et al., 1981; Schwab, 2000; Serbedzija et al.,

1990; Teitelman et al., 1982). Secondly, neuroblastoma cells share morphological and molecular characteristics with sympathoadrenal precursor cells. For example, they both express the precursor cell markers nestin, the noradrenergic marker tyrosine hydroxylase (TH), GAP43 and the neuronal marker neuron specific enolase (NSE) (Gestblom et al., 1997; Hoehner et al., 1996). Thirdly, neuroblastoma cells induced to differentiate with retinoic acid, for example, can adopt a sympathetic neuron-like phenotype (Kaplan et al., 1993). Furthemore, neuroblastoma almost never occurs in adults. It is therefore likely that neuroblastomas arise from an undifferentiated sympathetic neuron precursor cell. As such, neuroblastomas are often considered to be the transformed counterpart of sympathetic neurons.

1.3 Programmed cell death in neuroblastoma

Regulation of cell death is crucial for the development and maintenance of many mammalian tissues, including the nervous system. For example, neuronal progenitors are generated in excess during development, and programmed cell death (PCD) is implicated in the removal of those progenitor cells that fail to differentiate into neurons, or fail to receive sufficient amount of trophic factors (Miller et al., 2000). The best studied PCD is apoptosis, which is morphologically characterized by reduction of cellular volume, chromatin condensation, DNA fragmentation, membrane blebbing and apoptotic bodies (Thornberry and Lazebnik, 1998). These characteristics are mainly consequences of the activation of a special subset of cysteine-dependent aspartate-specific proteases called caspases, which are synthesized as inactive pro-forms that become activated following proteolytic processing (Thornberry and Lazebnik, 1998). For example, activation of caspase-3 results in the proteolytic processing of several downstream targets including the poly(ADP-ribose)polymerase (PARP) and the inhibitor of caspase-activated DNAse (ICAD), the latter being implicated in the regulation of DNA degradation (Nagata, 2000). Recent studies have identified two general signaling pathways capable of activating caspases. One is the receptor-mediated apoptotic pathway, which is triggered by the activation of a death receptor, such as the tumor necrosis factor receptor family member Fas (Budihardjo et al., 1999). The second is the mitochondrial pathway, which results in the release of mitochondrial cytochrome c

to trigger caspase activation (Budihardjo et al., 1999). Upstream of these two signaling cascades lies the tumor suppressor protein, p53, which is a transcription factor that modulates the expression of many genes implicated in the regulation of these two apoptotic pathways. Some of these genes include Bax (Miyashita and Reed, 1995), PUMA (Nakano and Vousden, 2001) NOXA (Oda et al., 2000), APAF-1 (Moroni et al., 2001) and Fas (Zhao et al., 2000). Inactivation of apoptosis impedes the capacity of cells to die, and this is frequently observed in human cancers (Hanahan and Weinberg, 2000). Since neuroblastoma likely arises from a sympathetic neuron precursor cell that failed to appropriately differentiate during normal development and failed to apoptose in consequence to this differentiation defect, it could be hypothesized that like many other types of human tumors, neuroblastoma cells become resistant to cell death due to inactivation of key components of their apoptotic machinery. Most studies characterizing cell death pathways in neuroblastoma have focused on chemotherapeutic drug-induced apoptosis. Thus, in this introduction I will review data describing the possible PCD signaling cascade induced by these drugs (figure 1).

p53 plays a crucial role in induction of apoptosis

p53 plays a crucial role in both neuronal and cancer cell death. First, postnatal sympathetic neurons deprived of their obligate survival factor, NGF (Nerve Growth Factor), up-regulate p53 prior to apoptosis (Aloyz et al., 1998). Second, expression of a dominant interfering p53 family member, such as ΔNp73, or functional ablation of p53 confers resistance to cell death of sympathetic neurons in absence of NGF (Pozniak et al., 2000) (Aloyz et al., 1998). Finally, human tumour cells that become resistant to cell death often have p53 deletion or mutations (Levine, 1997). Taken together with the capacity of p53 to induce transcription of pro-apoptotic genes, these observations suggest an important role for p53 in induction of neural cell apoptosis.

Interestingly, primary neuroblastomas taken from patients that have not yet undergone therapy express wild type p53 (Imamura et al., 1993; Komuro et al., 1993; Vogan et al., 1993). Although p53 is frequently sequestered in the cytoplasm of neuroblastoma cells away from its transcriptional targets (Moll et al., 1995; Moll et al., 1996), p53 is crucial for cell death induced by the chemotherapeutic drugs doxorubicin,

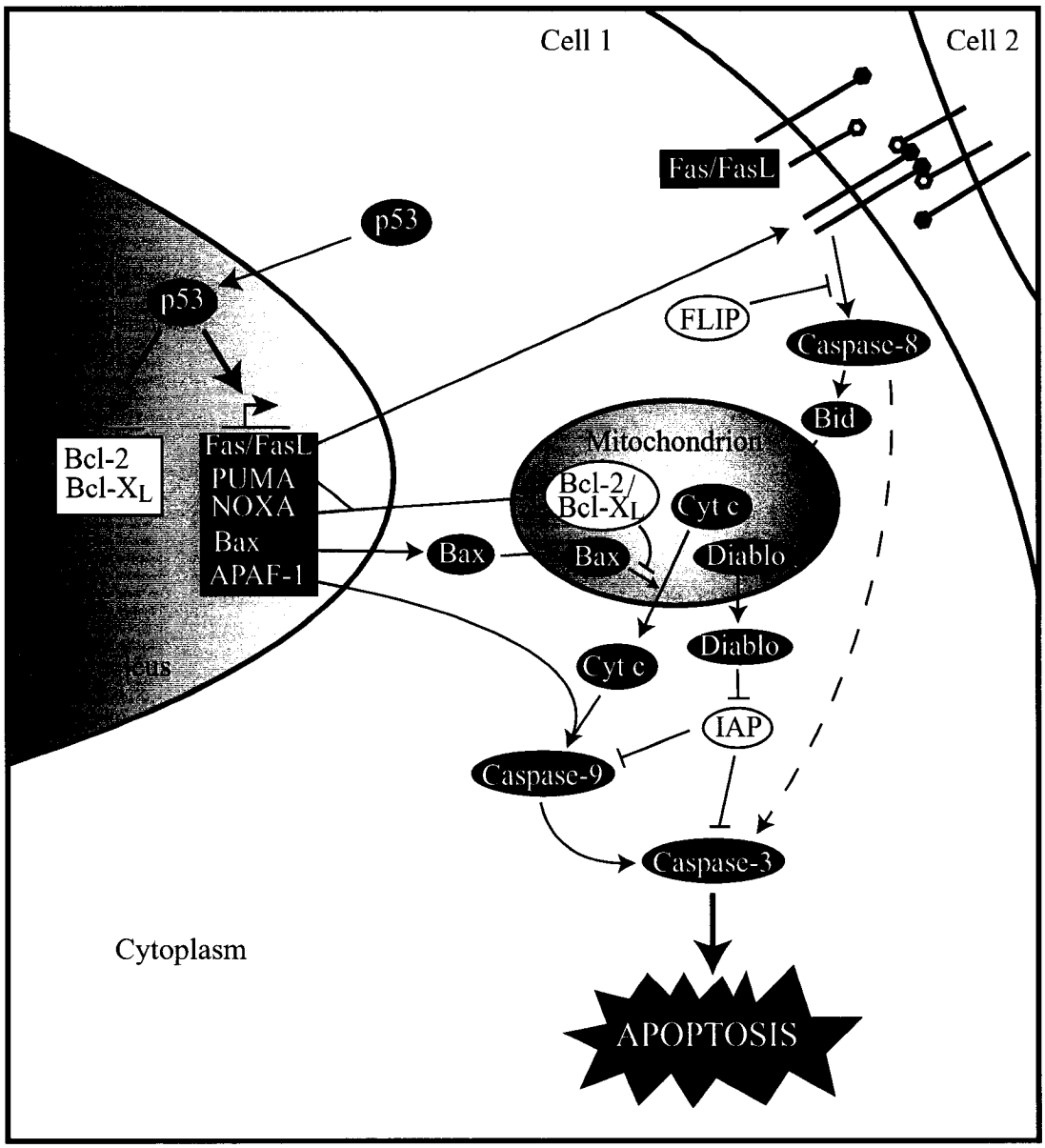


Figure 1.1: Schematic of cell death pathways potentially induced by common chemotherapeutic drug in neuroblastoma cells. The pro- and anti-apoptotic proteins are shown in black and white, respectively. For references and detailed description see the text.

cisplastin, and etoposide. Treatment with these drugs induces the up-regulation of p53 protein levels (Fulda et al., 1997). Furthermore, inactivation of p53 by overexpressing a dominant negative p53 or the papillomavirus 16E6 gene product, which induces p53 degradation, prevents doxorubicin- and etoposide-induced cell death (Cui et al., 2002). In addition, multi-drug resistant neuroblastoma cell lines lack expression of functional p53, either due to p53 mutation or due to high expression of the endogenous p53 protein repressor, MDM2 (Keshelava et al., 2001; Tweddle et al., 2001). Although the mechanism by which p53 becomes translocated from the cytoplasm to the nucleus is still unknown, these observations support the hypothesis that p53 plays a crucial role in chemotherapeutic drug-induced neuroblastoma cell death.

Erk1/2 are upstream of p53

The mechanism by which chemotherapeutic drugs activate p53 is not yet completely understood. Recently, two groups implicated the MAP kinases ERK1 and 2 in the up-regulation of p53 following cisplatin treatment. In these studies, the requirement for ERK1/2 was shown using the pharmacological inhibitor PD98059, which inhibits MEK, the kinase that activates ERK1/2. PD98059 treatment prevented the increase in p53 levels and the death of neuroblastoma cells treated with cisplatin (Park et al., 2001; Woessmann et al., 2002). However, several questions remain to be answered. How does cisplatin induce the activation of ERK1/2? Is expression of a dominantly active MEK sufficient to induce up-regulation of p53 and cell death in neuroblastoma? Finally, how does ERK1/2 cause the up-regulation of p53? The finding that ERK1/2 are involved in p53 up-regulation was not expected, since sympathetic neurons induced to apoptose following trophic factor deprivation do not require ERK1/2 activity to up-regulate p53 levels. Instead, it is the activation of two pro-apoptotic signal transduction cascades, (1) MLK-ASK-MEKK4/7-JNK-(c-jun)-p53-BIM/BAX-caspase-9/3 (reviewed by (Miller et al., 2000) and (Kanamoto et al., 2000; Putcha et al., 2001; Whitfield et al., 2001; Xu et al., 2001)) and (2) Cdk4/5-pRB-p19ARF-p53 (Park et al., 1996; Park et al., 1997), that converge to and increase p53 expression levels.

p53 pro-apoptotic gene targets

Pro-apoptotic p53 gene targets such as Bax (Miyashita and Reed, 1995), PUMA (Nakano and Vousden, 2001), NOXA (Oda et al., 2000), APAF-1 (Moroni et al., 2001), and Fas (Zhao et al., 2000) can be classified into two subgroups, based upon their involvement in either receptor-mediated (Fas) or mitochondria-mediated (Bax, PUMA, NOXA and APAF-1) apoptotic pathways. In neuroblastoma, Bax, Fas Ligand (FasL) and Fas are three p53 gene targets that have been confirmed to be expressed following treatment with chemotherapeutic drug *in vitro* (Fulda et al., 1997; Park et al., 2001). In addition, *de novo* gene transcription is required for doxorubicin, cisplatinum and etoposide to induce cell death in neuroblastoma, since the presence of cyclosporine, an inhibitor of transcription, is protective against the lethal effects of these drugs (Fulda et al., 1997). However, it remains to be determined whether inhibition of p53 is sufficient to prevent the upregulation of Bax, Fas and FasL by these agents.

Fas-mediated and mitochondria-dependent apoptotic pathways

In general, activation of Fas results in the activation of the initiator caspase, caspase-8, via the formation of the death-inducing signaling complex (DISC) (Budihardjo et al., 1999). Two downstream targets of caspase-8 are the effector caspase, caspase-3, and the pro-apoptotic Bcl-2 family member protein, Bid. In cells expressing high levels of caspase-8, active caspase-8 directly cleaves and activates caspase-3. However, in cells expressing lower levels of caspase-8, active caspase-8 induces caspase-3 activation indirectly, via the activation of the mitochondria pathway. This is achieved by the cleavage of Bid (which becomes tBid) by active caspase-8. Then tBid translocates to the mitochondria, where it induces the release of cytochrome c, which is one of the first events of the mitochondria apoptotic pathway. Once released, cytochrome c complexes with APAF-1 and pro-caspase-9, and in the presence of ATP/dATP, caspase-9 becomes activated (Li et al., 1997). Once active, caspase-9 activates capase-3 by proteolytic cleavage. The mitochondria-dependent apoptotic pathway can be regulated at several levels. One point of regulation is at the level of cytochrome c release, which can be induced following the translocation of Bax from the cytoplasm to the mitochondrial membrane (Wu and Deng, 2002). The action of Bax can be counteracted by the anti-

apoptotic Bcl-2 family member proteins Bcl-2 and Bcl-X_L which are present at the mitochondrial membrane (Budihardjo et al., 1999; Park and Hockenbery, 1996). However, the up-regulation of the pro-apoptotic Bcl-2 family members, Bax, PUMA, NOXA, Bad, and BIM, which can bind to Bcl-2 and Bcl-X_L, results in the neutralization of the anti-apoptotic function of Bcl-2 and Bcl-X_L (Budihardjo et al., 1999; Wu and Deng, 2002). As a consequence, Bax becomes derepressed and cytochrome c can be released. Therefore, the relative levels of pro- and anti-apoptotic proteins at the mitochondria regulate the activation of the mitochondrial apoptotic pathway. A second regulatory mechanism is at the level of caspase activation. The activity of caspase-3,-7 or -9 can be blocked upon binding to IAP (inhibitor-of-apoptosis protein) family members (Fesik and Shi, 2001; Nicholson, 2001). This inhibitory action of IAP can be counteracted by the Smac/DIABLO proteins, which are released from mitochondria following induction of apoptosis (Fesik and Shi, 2001; Nicholson, 2001). As discussed below, many of the proteins implicated in the Fas-mediated and mitochondria-dependent apoptotic cell death pathways may be involved in chemotherapeutic drug-mediated PCD in neuroblastoma.

Apoptotic pathways induced by chemotherapeutic agents

Treatment of neuroblastoma cells with chemotherapeutic drugs induces cell death in a caspase-dependent manner, since caspase inhibitors block the lethal effect of these drugs (Fulda et al., 1997; Yuste et al., 2002). As discussed above, two pathways capable of inducing caspase activation are the Fas-mediated and the mitochondria-dependent pathways. In neuroblastoma cells, both of these pathways are important for drug-induced apopotosis. This has been supported by several observations. For example, Fas expression becomes up-regulated in a *de novo* transcription-dependent manner following treatment with doxorubicin, cisplatin or etoposide (Fulda et al., 1997). The mechanism by which Fas is activated in this system remains to be determined, but it could be speculated that Fas might interact with its ligand, FasL, on neighboring cells. Alternatively, upregulation of Fas may be sufficient to induce its spontaneous trimerisation and activation (Nagata, 1997). In addition, drug-induced apoptosis is largely reduced in the presence of Fas function-blocking antibody. Similarly, a

neuroblastoma cell line resistant to Fas-mediated death is more resistant to drug-induced apoptosis (Fulda et al., 1997). Furthermore, antisense inhibition of c-FLIP, an endogenous inhibitor of caspase-8, sensitizes neuroblastoma cells to Fas-induced cell death (Poulaki et al., 2001). However, it remains to be determined whether the downregulation of c-FLIP can increase the sensitivity of neuroblastoma cells to chemotherapeutic drugs. The mitochondrial apoptotic pathway is also important for drug-induced apoptosis. For example, the treatment with doxorubicin, etoposide or staurosporine induces caspase-9 activation and cell death, effects that are blocked by overexpressing a dominant negative caspase-9 (Cui et al., 2002; Teitz et al., 2002). In addition, over-expression of Bcl-2 or Bcl-X_L prevents staurosporine-induced cell death (Yuste et al., 2002). Since both Fas and mitochondrial pathways are activated by chemotherapeutic drugs, the inhibition of which diminishes the lethal effects of these drugs, it could be suggested that these pathways are organized linearly and not in parallel. In support of this hypothesis, antisense inhibition of the pro-survival protein Bcl-2, which is part of the mitochondrial pathway, potentiates Fas-induced cell death of neuroblastoma cells (Poulaki et al., 2001). In addition, simultaneous inhibition of Bcl-2 and c-FLIP enhanced this effect in a synergic manner (Poulaki et al., 2001). In contrast to this hypothesis, a neuroblastoma cell line deficient in caspase-8 expression can be induced to apoptose following treatment with doxorubicin (Rebbaa et al., 2001), suggesting that caspase-8 is not necessary for drug-induced apoptosis. This discrepancy can be explained by the finding that chemotherapeutic drugs such as cisplatin induce the accumulation of the pro-apoptotic protein Bax (Park et al., 2001), which can directly activate the mitochondrial apoptotic pathway. Therefore, it could be hypothesized from these results that Fas and caspase-8 function upstream of the mitochondrial pathway, which might be directly activated, possibly following the upregulation of Bax, in the absence of a functional Fas/Caspase-8 pathway.

Mechanisms of apoptosis inhibition in unfavorable neuroblastoma

The apoptotic machinery in neuroblastoma tumors with favorable prognosis appears to be functional, since they respond to chemotherapy and can spontaneously regress. In contrast, neuroblastoma tumors with unfavorable prognosis are resistant to these drugs

and may have defects in apoptotic pathways. In this group of tumors, p53 is frequently sequestered in the cytoplasm, away from its nuclear transcriptional targets (Moll et al., 1995; Moll et al., 1996). Mutant or deleted p53, which confers resistance to therapeutic drugs, is also frequently observed in recurrent, drug-resistant neuroblastoma tumors (Imamura et al., 1993). In addition, several groups have reported that expression of caspase-8 is reduced or absent in 20 to 56 percent of primary neuroblastoma and in 50 to 70 percent of neuroblastoma cell lines, which may increase the sensitivity threshold for certain chemotherapeutic drugs (Banelli et al., 2002; Eggert et al., 2001; Takita et al., 2001). Further defects have been observed. For example the anti-apoptotic proteins Bcl-2, Bcl-X_L (Dole et al., 1995) and the IAP family member survivin (Islam et al., 2000) are often expressed at high levels in neuroblastoma, increasing the resistance of the tumor cells to apoptosis. Taken together, these observations suggest that alterations in apoptotic pathways are frequent in neuroblastoma and can increase the threshold for apoptosis. However, are these modifications responsible for the difference between poor and favorable prognosis neuroblastoma?

This question is related to two other important questions in the neuroblastoma field: What are the molecular differences between favorable and unfavorable neuroblastoma, and can an aggressive neuroblastoma be converted into a therapeutically responsive, more favorable-outcome tumor? One of the major approaches used in the cancer field to address these questions has been to 1) identify molecular markers that correlate with good or poor prognosis tumors, 2) determine the significance of the molecular markers in neuroblastoma biology, 3) ascertain how the molecular markers mediate their biological effects, and 4) develop new therapies based upon an understanding of how a given marker works at the molecular level.

1.4 Prognosis markers for neuroblastoma

A number of correlative markers for neuroblastoma have been identified during the past two decades, including genomic abnormalities, overexpression of proto-oncogenic proteins and the expression of neurotrophin receptors. Genomic abnormalities such as deletions at the distal p arm end of chromosome 1 (Brodeur et al., 1981; Caron et al., 1996; Fong et al., 1989; Gehring et al., 1995; Maris et al., 2000; Martinsson et al.,

1995; White et al., 1995; White et al., 2001), gain at the long arm of chromosome 17 (17q) (Bown et al., 1999; Caron, 1995), diploidy (Kaneko et al., 1990; Kaneko et al., 1987; Look et al., 1991), overexpression of the proto-oncogene protein MYCN (Brodeur et al., 1984; Seeger et al., 1985), high levels of telomerase activity (Hiyama et al., 1995; Reynolds et al., 1997; Streutker et al., 2001), and expression of the brain-derived neurotrophic factor (BDNF) receptor TrkB (Hoehner et al., 1995) are associated with poor clinical outcomes. In contrast, near triploidy (Kaneko et al., 1990; Kaneko et al., 1987), low expression of MYCN and high expression of the nerve growth factor (NGF) receptor TrkA (Hoehner et al., 1995; Nakagawara et al., 1992) are markers of favorable outcomes. Among these markers, only a few have been examined for their effects on the phenotype or molecular characteristics of neuroblastoma. The most extensively studied markers in this regard are TrkA, TrkB and N-myc.

1.5 Biological role of N-myc in neuroblastoma

The transcription factor and proto-oncogene N-Myc, encoded by the *MYCN* gene, regulates genes implicated in cell proliferation and apoptosis, and directly binds to pRb, an important cell cycle regulator (Rustgi et al., 1991). N-Myc is required for the development of several organs, including parts of the nervous system, as N-Myc homozygous null mice die at embryonic day 11.5 and have fewer numbers of neurons in the peripheral ganglia (Sawai et al., 1993; Stanton and Parada, 1992; Stanton et al., 1992). In these mice, the decrease in cerebellar neurons is a consequence of a reduction in proliferation rather than an increase in cell death (Knoepfler et al., 2002). The proliferative and anti-apoptotic role of N-Myc in neuronal cells is further supported by a recent report showing that overexpression of N-Myc protein in mature post-mitotic sympathetic neurons using a recombinant adenoviral vector induces cell cycle entry without induction of cell death, which normally occurs when these neurons are caused to cycle (Wartiovaara et al., 2002). However, those neurons overexpressing N-Myc did not undergo cell division, suggesting than in mature neurons, overexpression of N-Myc is not sufficient to induce mitosis. During mouse development, cytoplasmic sequestration of N-Myc, and thus its inactivation, has been implicated in neurotrophin-induced cell cycle arrest of neuroblasts (ElShamy et al., 1998). Therefore, the main biological

function of N-Myc during development may be to keep progenitor cells from exiting the cell cycle.

In neuroblastoma, N-Myc amplification, ranging from 3 to 300 fold, is observed in approximately 25% of patients (Bown, 2001; Schwab et al., 1983; Seeger et al., 1985), and correlates with advanced stage and poor outcome (Brodeur et al., 1984; Seeger et al., 1985). The potential roles of N-Myc in neuroblastoma biology have been examined by several groups. One study showed that two neuroblastoma cell subclones generated from a parental cell line showed different efficiencies to grow in soft agar according to their expression levels of N-Myc (Hino et al., 1989). The subclone with the highest levels of N-Myc was better able to grow in soft agar and to cause tumors when injected in nude mice than the subclone expressing lower levels of N-Myc (Hino et al., 1989). Similarly, suppression of N-Myc expression in neuroblastoma using anti-sense oligonucleotides has been shown to induce cell cycle arrest and block cell growth, but cell differentiation was not induced (Larcher et al., 1992; Sun et al., 2002). These results suggest that one function of high N-Myc levels in neuroblastoma is to keep the tumor cells in a proliferative state. The involvement of N-Myc in neuroblastoma pathology is further supported by the observation that transgenic mice overexpressing the *MYCN* gene under the tyrosine hydroxylase promoter develop "small round cell" tumors similar to human neuroblastomas (Weiss et al., 1997). These tumors developed in the abdominal and thoracic paraspinous regions, which are where human neuroblastomas are localized. At the cellular level, these tumor cells exhibited varying degrees of neuronal differentiation, expressed the neuronal markers synaptophysin and NSE, and had chromosomal abnormalities (Weiss et al., 1997). In contrast to human patients, these mice developed tumor masses only at older ages (from 3 to 6 months old) and bone metastases, a characteristic of aggressive neuroblastoma were not observed. Although these mice do not show all of the characteristics of aggressive N-Myc amplified tumors, they are currently the only animal model for neuroblastoma.

While these studies suggest that N-Myc overexpression plays an important role in neuroblastoma progression, it is important to keep in mind that overexpression of N-Myc occurs in only 25% of cases. In addition, N-Myc overexpression is not always

present in all fatal neuroblastoma cases (Brodeur, 2003). Therefore, it was important to identify additional prognosis markers predictive for poor and good outcomes.

1.6 Neurotrophin receptors and neuroblastoma tumours

The expression of two members of the Trk family of receptor tyrosine kinases, TrkA and TrkB, has been shown to be prognostic in neuroblastoma. The Trk family is comprised of three members, TrkA, TrkB and TrkC. The ligands for the mammalian Trks are the neurotrophins Nerve Growth Factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4. NGF and NT-3 bind to TrkA, BDNF and NT-4 are the preferred ligands for TrkB, and NT-3 binds TrkC (Barbacid, 1995). A second type of neurotrophin receptor, the p75 neurotrophin receptor (p75NTR), is a prototypic member of the tumor necrosis receptor family (Hempstead, 2002). p75NTR can bind all four neurotrophins, as well as their unprocessed "pro" forms (Lee et al., 2001). The expression of the neurotrophins and their receptors is crucial for the proper development of the mammalian nervous system. Mice deficient in the expression of any of the Trk receptors or their ligands show severe nervous system defects, such as neuronal loss (Farinas, 1999). In contrast, mice deficient in the p75NTR exhibit a delay in naturally occurring cell death of sympathetic neurons, loss of apoptosis in the basal forebrain cholinergic neurons (Naumann et al., 2002), and defects in sympathetic axon target innervation (Bamji et al., 1998; Kohn et al., 1999; Walsh et al., 1999).

In rodents, sympathetic neuron progenitor cells express TrkC, but not TrkB (Birren et al., 1993). As the cells mature, expression of TrkA is induced, while TrkC expression is down regulated (Birren et al., 1993; Fagan et al., 1996). TrkA expression coincides with cell cycle arrest of sympathetic neuroblasts and subsequently and throughout postnatal life, their survival or growth depends on NGF/TrkA signaling (Henderson, 1996; Verdi and Anderson, 1994).

TrkA expression in neuroblastoma

An important finding in the neuroblastoma field was the discovery by Brodeur's laboratory that high expression of TrkA correlates with favorable outcome in human neuroblastoma (Hoehner et al., 1995; Nakagawara et al., 1992; Nakagawara et al., 1993).

This result led to the examination of the effect of TrkA expression in cultured neuroblastoma cells which are derived from poor prognosis patients. As a consequence, many neuroblastoma cell lines do not express endogenous levels of TrkA. Those that do express TrkA respond to NGF by differentiating into neuronal-like cells (Chen et al., 1990). Similarly, overexpression of TrkA converts NGF non-responsive neuroblastoma cells into NGF-responsive cells (Lavenius et al., 1995; Matsushima and Bogenmann, 1993). Differentiated cells were assessed by neurite extension and increases in the expression of neuronal markers SCG10 and neurofilament NF-L (Lavenius et al., 1995; Matsushima and Bogenmann, 1993). Another important biological response induced by NGF in TrkA-overexpressing cells is the inhibition of cell growth (Lucarelli et al., 1997; Matsushima and Bogenmann, 1993; Poluha et al., 1995). The anti-proliferative effects induced by NGF and TrkA have also been assessed *in vivo*. Injection of TrkA-overexpressing neuroblastoma cells into nude mice resulted in the formation of tumors. Treatment of these tumors with NGF induced the *in vivo* differentiation into neural and schwann cells (Matsushima and Bogenmann, 1993).

Interestingly, expression of TrkA in primary tumors inversely correlates with the expression of N-Myc (Nakagawara et al., 1992). Thus, TrkA and N-Myc expression may antagonize other's expression. In support of this hypothesis, TrkA overexpression induced the down-regulation of N-Myc expression in an N-Myc-amplified neuroblastoma (Matsushima and Bogenmann, 1993). In addition, overexpression of N-Myc under the U3 LTR region of the Moloney murine leukemia virus inhibits TrkA-induced cell cycle exit and neuronal differentiation of neuroblastoma cell lines (Bogenmann et al., 1995). In this study, NGF treatment of TrkA overexpressing cells did not alter N-Myc expression levels. The mechanism whereby N-Myc and TrkA regulate each other's expression and function are not known, but it is possible that TrkA may down regulate N-Myc expression at the transcriptional level. It would be interesting to determine whether N-Myc expression could down-regulate endogenous levels of TrkA expression because the relative expression levels of TrkA and N-Myc might play a role in determining which of the two proteins will exert its biological effects (Eggert et al., 2000a), and whether a tumor will differentiate and regress or become malignant and metastasize.

These results suggest that TrkA expression may cause malignant neuroblastoma cells to become quiescent differentiated cells, which is consistent with it being a favorable prognosis marker. The anti-proliferative and differentiative effects of TrkA in neuroblastoma cells might explain why TrkA is a good prognosis marker for this type of tumor.

TrkB expression in neuroblastoma

In contrast to TrkA, expression of TrkB correlates with poor prognosis and N-myc amplification (Aoyama et al., 2001; Hoehner et al., 1995; Nakagawara et al., 1994). The expression of TrkB in neuroblastoma was unexpected, since it is not normally expressed in sympathetic neuron precursors (Birren et al., 1993). Its expression is also low in neuroblastoma cell lines (Kaplan et al., 1993), although it can be induced to high levels by retinoic acid treatment (Kaplan et al., 1993). The biological role of TrkB in neuroblastoma cells has been examined following TrkB transfection or retinoic acid treatment. TrkB mediates neuronal differentiation, but unlike TrkA, does not induce cell cycle arrest (Kaplan et al., 1993; Lucarelli et al., 1997). In addition, in contrast to TrkA, BDNF binding to TrkB mediates cell survival, invasiveness, proliferation and resistance to chemotherapeutic agents, the last characteristic being a feature of aggressive neuroblastomas (Ho et al., 2002; Matsumoto et al., 1995; Middlemas et al., 1999; Scala et al., 1996). These properties of TrkB might account for why TrkB is a poor prognosis marker for neuroblastoma.

Inhibition of either TrkB or one of its signaling cascades (section on Trk signaling cascade below) renders neuroblastoma cells more sensitive to chemotherapeutic agents (Ho et al., 2002; Jaboin et al., 2002). Since neuroblastoma cells can also express BDNF (Nakagawara et al., 1994), an autocrine BDNF/TrkB signaling may be generated which might confer the aforementioned biological properties characteristic of aggressive neuroblastomas.

Role of TrkC and p75NTR in neuroblastoma

The role of the remaining Trk receptor, TrkC, in neuroblastoma is not yet clear. TrkC expression in neuroblastoma correlates with low tumour grade and favourable prognosis,

but in most cases TrkC is co-expressed with TrkA (Brodeur et al., 1997; Svensson et al., 1997; Yamashiro et al., 1997; Yamashiro et al., 1996). No study has specifically determined whether primary neuroblastoma can only express TrkC in the absence of TrkA. It would be interesting to determine whether co-expression of TrkA and TrkC in neuroblastomas is a better indicator of prognosis than TrkA alone. Nevertheless, two independent *in vitro* studies in which TrkC was ectopically expressed by either transfection or retroviral infection showed that TrkC expression restored NT-3 responsiveness in these cells. Treatment of the TrkC-expressing cells with NT-3 induced cell differentiation, but not growth arrest (Edsjo et al., 2001; Yamashiro et al., 1997). The data from these studies suggest that TrkC induces a biological response similar to TrkB, rather than TrkA, with respect to cell differentiation and proliferation.

To clarify the role of TrkC in neuroblastoma biology, further experiments will be required. For example, TrkC could be overexpressed in neuroblastoma cells to determine whether TrkC will potentiate TrkA biological responses such as differentiation, cell cycle arrest and down-regulation of N-Myc. Alternatively, TrkC expression could be suppressed by expressing a dominant negative TrkC to determine whether the loss of TrkC activity diminishes the biological responses induced by TrkA activation.

p75NTR

The expression of p75NTR in neuroblastoma does not significantly correlate with tumour grade (Brodeur et al., 1997). The role of p75NTR in neuroblastoma is not yet evident. Two studies have reported that overexpression of p75NTR in the absence of neurotrophin (NGF) addition induces the death of neuroblastoma cell lines (Bunone et al., 1997; Eggert et al., 2000c). However, these studies reported conflicting results concerning the role of p75NTR in the presence of neurotrophins. While Bunone *et al.* showed that NGF prevented p75NTR-induced cell death (Bunone et al., 1997), Eggert *et al.*, observed that TrkA overexpression, but not NGF, prevented the death of the p75NTR-expressing cells (Eggert et al., 2000c). Two other studies showed that in the presence of NGF, p75NTR prevented cell death induced as a consequence of cell cycle arrest caused either by the anti-mitotic drug neocarzinostastin (Cortazzo et al., 1996) or following the activation of a chimeric TrkA receptor (Lachyankar et al., 2003). The

different results could be explained by the use of different neuroblastoma cell lines, the cell cycle status of these cells when they were assayed, or the relative levels of p75NTR and TrkA overexpression in each study. All but one of these studies assessed the action of over-expressed p75NTR, rather than endogenous p75NTR. To determine whether p75NTR in the presence or absence of different neurotrophins will modulate cell survival, proliferation or differentiation, experiments using different cell lines will be required, with cells expressing only endogenous p75NTR, TrkA, or co-expressing both receptors.

1.7 Master's project

During the last decade, several prognostic markers have been associated with different stages of neuroblastoma. Among these, the TrkA and TrkB receptors, despite being 90% similar in their intracellular domains, correlate very differently with prognosis, with TrkA associated with good prognosis, and TrkB with poor prognosis. Recently, in our laboratory, we have observed a novel biological response induced by TrkA in neuroblastoma cells. The transient overexpression of TrkA, but not TrkB in two neuroblastoma cell lines as well as in primary neuroblastoma cells induced cell death. *For my Master' thesis, I decided to confirm this result, and determine how TrkA expression leads to cell death.* The induction of cell death by TrkA might explain why TrkA is a good prognosis marker for neuroblastoma. Since TrkA expression does not result in the death of non-transformed neural cell types, the identification of the signaling proteins used by TrkA to mediate cell death may provide novel targets for the discovery of drugs to specifically induce neuroblastoma regression.

1.8 Trk signal transduction in sympathetic neurons and neuroblastoma

Activation of the Trk receptors by their appropriate neurotrophin results in the activation of three major signal transduction cascades, the phosphoinositol-3-kinase (PI-3K)/Akt, the Ras-Raf-MEK1/2-ERK1/2 and the phospholypase C-γ (PLC-γ)/protein kinase C (PKC) pathways (Kaplan and Miller, 2000). These signal transduction cascades have been mainly studied in the rat pheochromacytoma cell line, PC12, and in several types of primary neurons. However, in neuroblastoma cells, the characterization of Trk

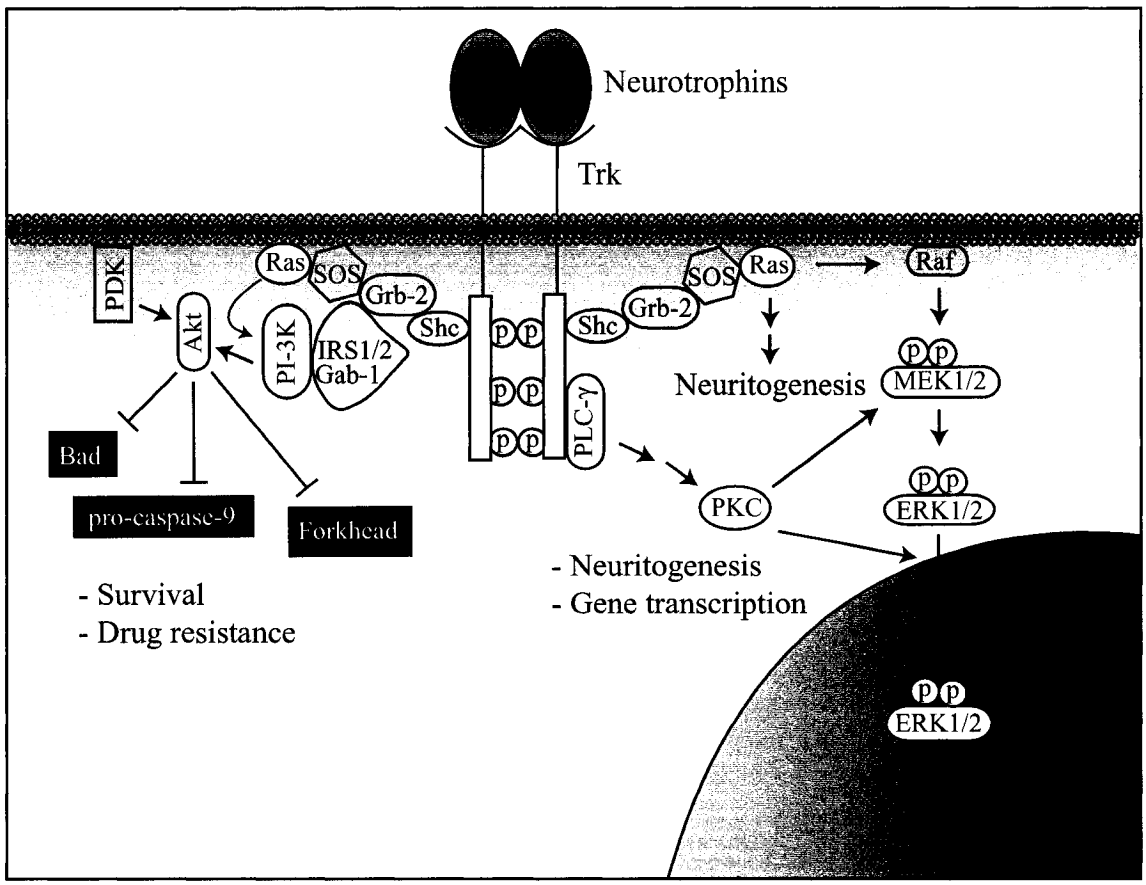


Figure 1.2: Model of Trk signaling cascades in neuroblastoma cells and their biological implications. Upon binding to neurotrophins, Trk receptors dimerize, auto-phosphorylate, and recruit several adaptor proteins (Shc, FRS-2, SH2-B and APS-1) and enzymes such as PLC-γ. In neuroblastoma some of these proteins (in white) were studied and are discussed in the text. The pro-apoptotic proteins are shown in black boxes.

signaling cascades has just begun. In this section I will compare recent data on Trk signal transduction in neuroblastoma cells with the three well characterized Trk signal transduction cascades in sympathetic neurons.

Upon binding to NGF, the TrkA receptor dimerizes and its cytoplasmic kinase domain becomes activated, resulting in the transphosphorylation of the receptor at specific cytoplasmic tyrosine residues (Kaplan and Miller, 1997). The phosphorylation of these residues is important to fully activate the kinase domain (Cunningham et al., 1997) and to recruit to the receptor several proteins implicated in TrkA signal transduction (Kaplan and Miller, 1997). Some of the recruited proteins include the signaling proteins PLC-γ, and the adaptor proteins Shc, FRS-2, SH2-B and APS-1 (Kaplan and Miller, 1997; Loeb et al., 1994; Qian et al., 1998; Rui et al., 1999). The binding of Shc, and its subsequent phosphorylation by TrkA, results in the activation of two of the most important proteins in NGF signal transduction with regard to survival and growth: the small GTP-binding protein Ras and phosphatidylinositol-3-kinase (PI-3K) (Kaplan and Miller, 2000). Thus, Ras, PI-3K and PLC-γ are part of three important signal transductions activated by TrkA in sympathetic neurons (Kaplan and Miller, 2000).

The PLC-γpathway

Activation of PLC-γ results in the hydrolysis of phosphatidylinositol-(4,5)-biphosphate (PIP_2), located at the cytoplasmic membrane, generating the two second messengers, diacylglycerol (DAG) and inositol-tri-phosphate (IP_3). DAG activates DAG-regulated protein kinase C (PKC) isoforms such as PKC-δ (Liu and Heckman, 1998), while IP_3 is a potent intracellular calcium regulator. In hippocampal neurons, TrkB-mediated PLC-γ activation is crucial for long-term potentiation (LTP), as mice expressing a mutant TrkB receptor lacking the PLC-γ binding site show defects in LTP induction (Minichiello et al., 2002). However, in sympathetic neurons and PC12 cells, the role of the PLC-γ signaling pathway is not known, as the PLC-γ binding site of TrkB and TrkA is dispensable for TrkB-induced survival and growth of sympathetic neurons transiently overexpressing TrkB (Atwal et al., 2000). PLC-γ is also dispensable for TrkA-induced survival and axonal growth of PC12 cells expressing a TrkA receptor mutated at the

PLC-γ binding site. However, PLC-γ is required for NGF-induced peripherin expression in these cells (Loeb et al., 1994). Taken together, these results suggest that the PLC-γ pathway is involved in gene expression, but not in the survival and axonal growth of PC12 cells and sympathetic neurons.

In neuroblastoma, most studies have focused on the role of the downstream PLC-γ effector, PKC, rather than PLC-γ itself. Using the SH-SY5Y human neuroblastoma cell line stably transfected with TrkA, the Nånberg laboratory showed that the inhibition of PKC, but not MEK, using the pharmacological inhibitors GF109203 and Ro-32-0432, prevented TrkA-induced neurite outgrowth (Olsson et al., 2000). This suggests that the PLC-γ/PKC pathway, but not the MEK/ERK1/2 pathway is required for TrkA-induced neuritogenesis. In addition, activation of TrkA by NGF in the presence of these PKC inhibitors reduced phosphorylated ERK accumulation in the nucleus without affecting the overall ERK phosphorylation levels (Olsson et al., 2000). This suggests that the PLC-γ/PKC and MEK/ERK pathways can collaborate to mediate some of TrkA-mediated cellular responses. In addition, the activity of some PKC isoforms is implicated in neuroblastoma survival and resistance to chemotherapeutic drugs. Svensson and Larsson reported that inhibition of the PKC-β isoforms using a pharmacological inhibitor decreased the growth of neuroblastoma cell lines, and increased the susceptibility of the SK-N-BE(2) neuroblastoma cell line to chemotherapeutic drug treatment (Svensson and Larsson, 2003). It is important to note that these experiments were performed in the absence of Trk activity, as no neurotrophins were added to the cultures. It can be hypothesized that TrkA and TrkB might activate different PKC isoforms as TrkB, but not TrkA, has been reported to induce drug resistance. Therefore it would be interesting to determine whether inhibition of PKC can decrease the protective effects of TrkB. However, to confirm the role of PLC-γ/PKC pathway in neuritogenesis and survival of neuroblastoma cells, it would be necessary to either suppress PLC-γ expression using anti-sense RNA, small interfering RNA (siRNA) or express a mutant Trk receptor lacking the PLC-γ binding site in order to determine whether the loss of Trk-induced PLC-γ activity decreases neuritogenesis and survival. Recently, Eggert *et al.* used the TrkA mutant strategy to determine the role of different TrkA substrate binding sites in neuroblastoma cells. However, in this study

(Eggert et al., 2000b), activation by exogenous NGF of ectopically expressed wild type TrkA resulted in cell proliferation rather than cell growth inhibition that has been observed and reported by several other groups (Lucarelli et al., 1997; Matsushima and Bogenmann, 1993; Poluha et al., 1995). Further experiments using TrkA and TrkB mutant receptors will be therefore be required to determine the role of Trk-induced PLC-γ activity in neuroblastoma cells.

The MEK/ERK signaling cascade

The NGF-induced recruitment of Ras to the membrane and its subsequent activation by SOS results in the activation of the Raf-1/MEK1/2/ERK1/2 signaling cascade (Kaplan and Miller, 2000). In sympathetic neurons, which endogenously express TrkA, but not TrkB, this pathway is dispensable for TrkA-induced survival (Mazzoni et al., 1999; Vaillant et al., 1999). However, the active MEK/ERK1/2 pathway has a protective role in sympathetic neurons under certain stresses, such as treatment with the pro-apoptotic compound cytosine arabinoside (Anderson and Tolkovsky, 1999). Interestingly, TrkB seems to mediate survival differentially from TrkA, since in sympathetic neurons induced to transiently express TrkB, inhibition of MEK/ERK1/2 pathway decreases BDNF-mediated survival (Atwal et al., 2000). Although the mechanism by which MEK exerts its pro-survival effect is not fully understood, in PC12 cells, Liu *et al* showed that the activation of ERK1/2 results in the up-regulation of the anti-apoptotic gene Bcl-2 (Liu et al., 1999). The MEK/ERK1/2 signaling pathway is also implicated in neuronal differentiation. As reviewed by Kaplan and Miller, sustained activation of the MEK/ERK1/2 pathway is necessary, but not sufficient to induce neurite outgrowth in several primary neuronal cell types and to induce the differentiation of PC12 cells (Kaplan and Miller, 1997). These observations suggest that the MEK/ERK1/2 signaling pathway is dispensable for TrkA-mediated survival, but is protective against certain neuronal stresses and is involved in neuronal growth. However, activation of this pathway could be modulated such that it could affect survival mediated by ectopically expressed TrkB, which is frequently expressed in aggressive neuroblastoma cells.

In neuroblastoma, the Ras-MEK-ERK1/2 pathway is implicated in gene transcription and neuritogenesis, depending on which Trk receptor is expressed. For

example, inhibition of PKC, but not MEK/ERK1/2, abrogates NGF/TrkA-induced neurite formation (Olsson and Nanberg, 2001). However, in a cell line overexpressing TrkA, expression of a dominantly active Ras (V12Ras), but not a dominantly active MEK, induces serum-responsive element (SRE) responsive genes such as Fos, and also induces neurite formation (Olsson and Nanberg, 2001). This suggests that sustained Ras activation is sufficient and PKC activation is required for neurite outgrowth, but the activation of the MEK/ERK1/2 cascade is neither sufficient nor required for neuritogenesis in these cells. However, it remains to be determined whether inhibition of ERK or PKC activity prevents dominantly active Ras from inducing neurite outgrowth. In contrast, in neuroblastoma cells treated with retinoic acid (RA) to induce differentiation and TrkB expression, treatment with a MEK inhibitor prevented BDNF/TrkB-induced neuritogenesis (Encinas et al., 1999). These observations suggest that the biological response elicited by the MEK/ERK1/2 signaling pathway depends on either the cellular context (differentiated or undifferentiated) or the Trk receptor expressed by neuroblastoma cells. In addition, the activation of the MEK1/2-ERK1/2 pathway is required in both TrkA and TrkB expressing cells to induce transcription of genes such as GAP-43 and c-fos (Encinas et al., 1999; Olsson and Nanberg, 2001).

PI-3K/Akt signal transduction

A third signal transduction cascade activated by the Trk receptor, and one of the most important for Trk-mediated survival of sympathetic neurons and PC12 cells, is the PI-3K/Akt pathway (Kaplan and Miller, 2000). PI3-kinase is activated by recruitment to Ras and Gab-1 (Holgado-Madruga et al., 1997; Kaplan and Miller, 2000; Mazzoni et al., 1999), resulting in the phosphorylation of phosphatidylinositol-bi-phosphate (PIP_2) to form PIP_3 at the inner surface of the cytoplasmic membrane (Vivanco and Sawyers, 2002). PIP_3 binds, recruits to the cell membrane, and alters the conformation of the serine-threonine kinase Akt (also known as protein kinase B, PKB) as well as the Akt activator 3-phosphoinositide-dependent protein kinase-1 (PDK1). Phosphorylation of Akt by PDK1 and PDK2 results in Akt activation (Vivanco and Sawyers, 2002). Downstream targets of Akt include a number of pro-apoptotic proteins, including the Bcl-2 family member Bad (Datta et al., 1997), the transcription factor Forkhead (Brunet

et al., 1999), pro-caspase-9 (Cardone et al., 1998) and GSK-3 (Cross et al., 1995; Pap and Cooper, 1998). Bad, Forkhead and pro-caspase-9 are all important inducers of apoptosis, and are inactivated when phosphorylated by Akt. It is not surprising then that PI-3-kinase is responsible for up to 80% of neurotrophin-mediated cell survival of sympathetic neurons (Kaplan and Miller, 2000). PI-3K activity is also important for the local axonal growth of sympathetic neurons. Inhibition of PI3-K activity in axons inhibits axonal growth, while inhibition in cell bodies suppresses neuronal survival (Atwal et al., 2000). Therefore, in neuronal cells, PI-3K is important for both survival and axonal growth.

In neuroblastoma cells, the PI-3K/Akt pathway plays an important pro-survival role. For example, TrkB mediates the survival of RA-differentiated neuroblastoma cells in the absence of serum via the PI-3K/Akt pathway, since pharmacological inhibition of PI-3K blocks TrkB-mediated survival (Encinas et al., 1999). However, does TrkB protect neuroblastoma cells from *chemotherapeutic agents* via the PI-3K/Akt pathway? This question has been addressed by Jaboin et al., who showed that inhibition of PI-3K abrogates the protective activity of TrkB in cells treated with chemotherapeutic drugs (Jaboin et al., 2002). Thus the PI-3K/Akt signaling pathway is required for TrkB-induced drug resistance. Since TrkA can activate this signal transduction pathway, but cannot confer resistance to chemotherapeutic drugs, it can be hypothesized that TrkA and TrkB modulate the PI-3K/Akt signaling pathway differently, perhaps by directing Akt to different cellular targets.

In summary, these observations suggest that in neuroblastoma cells, TrkA mediates neurite outgrowth through PLC-γ/PKC, but not the MEK/ERK1/2 pathway, while TrkB mediates this event via the MEK/ERK1/2 pathway. A second difference between the signaling of these two receptors is that although both activate the PI-3K/Akt signaling pathway, only TrkB can confer chemotherapeutic resistance by that pathway.

1.9 Working hypothesis and objectives

Does TrkA induce the death of neuroblastoma cells, and if so, what is the molecular mechanism used by this receptor to mediate cell death? To address these questions, I

will determine whether TrkA induces cell death when overexpressed using recombinant adenovirus vectors. If TrkA induces cell death, I hypothesize that it does so by the following mechanisms. During development, TrkA is expressed at the time sympathoblasts exit the cell cycle to differentiate into sympathetic neurons (Birren et al., 1993). Those neuronal progenitor cells that fail to differentiate will undergo apoptosis (Miller et al., 2000). Therefore, two hypotheses that may not be mutually exclusive could be formulated. First, I hypothesize that TrkA overexpression induces cell death by activating the apoptotic machinery in undifferentiated neuroblastoma cells. This will be assessed by identifying and characterizing the apoptotic proteins in cells expressing TrkA. An alternative hypothesis is that TrkA, which is an anti-mitogenic receptor, might induce the death of constitutively cycling neuroblastoma cells by causing conflicting cell cycle signals. This will be assessed by examining the regulation of cell cycle repressors following TrkA expression.

Chapter II: Determination of TrkA-induced cell death signaling cascade

2.1 Introduction

One of the most intriguing biological characteristics of neuroblastoma is the correlation between the expression levels of the tyrosine kinase NGF receptor, TrkA, and patient prognosis. High expression of TrkA is observed in favorable tumors, while very low to negligible expression of TrkA is present in aggressive tumors. Re-introduction of TrkA expression in neuroblastoma cells and its subsequent activation by NGF leads to cell differentiation and cell cycle arrest, two biological responses consistent with the correlation of TrkA expression with favorable neuroblastoma tumors (see chapter I). Another biological event consistent with this correlation is the preliminary observation by Dr. LeSauteur in Dr. David Kaplan's laboratory that ectopic expression of TrkA in a primary neuroblastoma cell line and in several established neuroblastoma cell lines, results in the induction of cell death (unpublished data). This chapter of my Master's thesis focuses upon confirmation of the cell death-inducing action of TrkA in neuroblastoma cell lines and in determining the molecular mechanisms by which this occurs. To achieve these goals, TrkA was expressed in two neuroblastoma cell lines and cell death and the molecular effects of TrkA were assessed. For this study we used the human neuroblastoma cell lines LAN-1-15N (15N) and NGP, which express undetectable or very low levels of endogenous TrkA, respectively. In addition, these two cell lines overexpress the N-myc oncogene (Ciccarone et al., 1989; Schwab et al., 1983), and have no reported p53 mutations. To express TrkA in these cells we used an adenovirus vector, which allows the non-toxic, transient, and high expression of TrkA protein. In addition, this technique has the advantage over stable transfection of not selecting for a sub-population of neuroblastoma cells that might become resistant to TrkA-induced cell death. The biological and molecular responses induced by TrkA in these cells were monitored using standard survival and molecular assays.

In this chapter, we demonstrate that TrkA efficiently induces apoptosis in these neuroblastoma cell lines, and that apoptosis may occur through a decrease in pro-survival proteins such as Bcl-2 and an increase in pro-apoptotic proteins such as p53 and

active caspase-3. These results suggest that one of the reasons that TrkA is a favorable prognostic marker for neuroblastoma is that high TrkA expression will result in the death of the tumor cells. Furthermore, the TrkA expressing adenovirus vector may be useful as a gene therapy reagent for the treatment of neuroblastoma.

2.2 Results

Generation of adenovirus expressing TrkA

To confirm the death inducing potential of TrkA, we used the previously generated recombinant TrkA-expressing adenovirus vector, TrkA Ad5, which expresses human TrkA tagged at its extracellular domain with the Flag epitope under a tetracycline (Tta) sensitive cytomegalovirus (CMV) promoter. To obtain a sufficient quantity of this adenovirus to perform all the experiments of this study, TrkA Ad5 was reamplified from the previously amplified TrkA Ad5 used by Dr. Lynne LeSauteur. After the processes of amplification and purification, we tested the virus for the presence of the wild type (WT) adenovirus gene E1A and E1B by PCR. This procedure is essential to ensure that during the amplification process, no virions have reverted to a WT phenotype by acquiring E1A and E1B genes in their genome, or that no WT adenovirus contamination occurred. The newly amplified TrkA Ad5 was positive for E1A and E1B genes (figure 2.1 lane 7). To determine the source of the contamination, we determined whether the TrkA Ad5 used for the amplification was also contaminated. As shown in figure 2.1 lane 6, TrkA Ad5 used to amplify the contaminated TrkA Ad5 and previously used by Dr. LeSauteur was contaminated with wild type (WT) Ad5 (figure 2.1 lanes 6 and 7). Since wild type Ad5 expresses genes that affect cell cycle progression and cell survival, we had to produce pure TrkA Ad5. Only then could we determine whether TrkA could induce the death of neuroblastoma cells.

To obtain a pure TrkA Ad5 we used two separate approaches. The first was to decontaminate the adenovirus that was re-amplified from the contaminated stock using a plaque assay technique. This assay allows the isolation of single plaque forming units, formed by one infectious virus particle. Virus particles isolated from thirty individual plaques were isolated, amplified and used to infected 293Tta cells, which stably expresses Tta and the adenovirus E1A and E1B regions that are missing in the

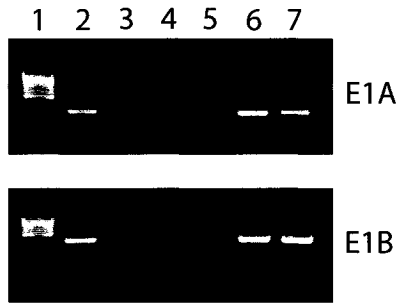


Figure 2.1: The newly purified TrkA adenovirus is free from E1A and E1B genes. Three different TrkA Ad5 were assayed for the presence of E1A and E1B genes by PCR. Lane 1: Ladder; lane2: wildtype contaminated Ad5, which was used as positive control; lane 3: Ad5 free of E1 genes that was used as negative control; lane 4; no template sample, which was also used as negative control; lane 5: pure TrkA Ad5 amplified from old Josee Wong's crude virus; lane 6, TrkA Ad5 used by Dr. LeSauteur; lane 7, TrkA Ad5 amplified from virus of lane 6.

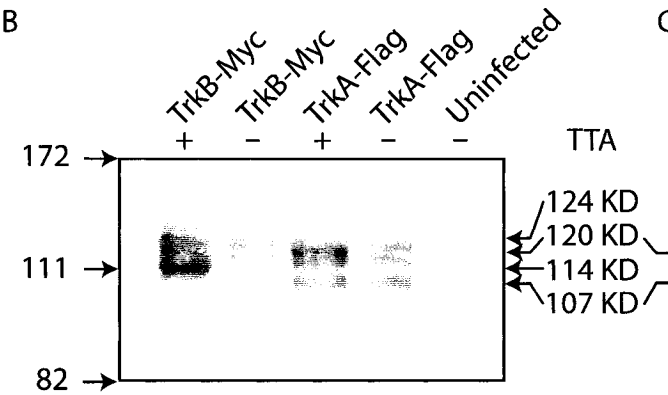
recombinant viral vector. The capacity of these amplified virus particles to express the TrkA-Flag protein was determined by Western blotting using an anti-Flag antibody. This technique revealed that none of the thirty isolated plaques contained virus particles capable of inducing TrkA-Flag expression in these cells, indicating that the re-amplified virus stock consisted predominantly of wild-type virus (data not shown). The second approach, which was successful, consisted of re-amplifying the TrkA Ad5 from lysates of 293 cells used for the original recombination event that created the TrkA A5 virus. Amplification and purification of this TrkA Ad5 yielded a high titer stock of TrkA Ad5 free of E1A and E1B WT Ad5 gene products, as assessed by PCR (figure 2.1 lanes 5). We next determined whether this pure TrkA Ad5 was capable of inducing TrkA expression.

We next determined the amount (or MOI) of TrkA Ad5 adenovirus that infected the maximum amount of cells in a tissue culture dish. Using standard immunostaining techniques, we assessed the expression of TrkA-Flag in TrkA Ad5 infected and uninfected NGP cells using anti-Flag. The per cent of infected cells was determined following 27 hours of infection with TrkA Ad5 in (figure 2.2A, in red) in TrkA Ad5-infected NGP, but not in uninfected cells. At a multiplicity of infection (MOI) of 50, we determined that the infection rate was approximately 50%. However, this number might be an underestimation of the actual infection rate; at later time points almost all TrkA Ad5-infected cells showed a rounded morphology and lacked processes, which contrasts with the flat morphology and the short processes observed in uninfected or control virus infected cells (see figures 2.4A, 2.8A and 2.9B). Based on these observations, we decided to use TrkA Ad5 at a MOI of 50 for all further experiments described in this study.

Since TrkA Ad5 encodes TrkA-Flag under a Tta-sensitive cytomegalovirus (CMV) promoter, we determined whether pure TrkA Ad5 requires the presence of the transcriptional activator Tta to induce TrkA expression. To achieve this goal, we used standard immunoprecipitation techniques using the pan-Trk antibody, 203A, to determine TrkA expression in lysates of NGP cells that were either infected with TrkA Ad5 alone or co-infected with TrkA Ad5 and Tta Ad5 for 36 hours (hrs). As a positive control for the Trk immunoprecipitation, we used cell lysates of NGP cells that were

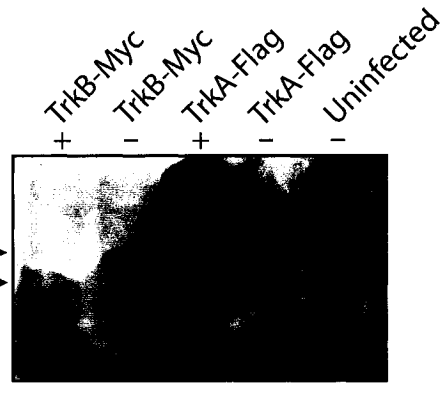
A

B



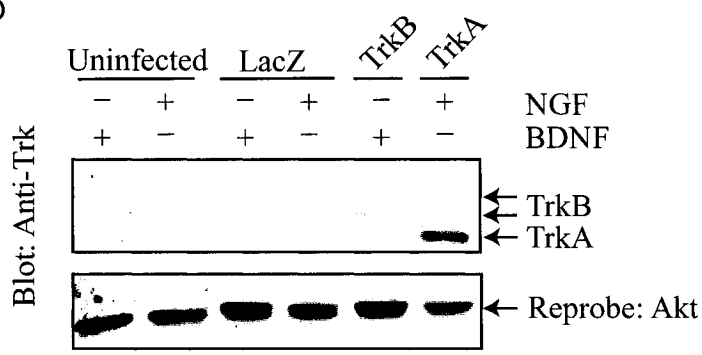
IP: Anti-Trk 203
Blot: Anti-Trk 203

C



Reprobe: Anti-Flag

D



E

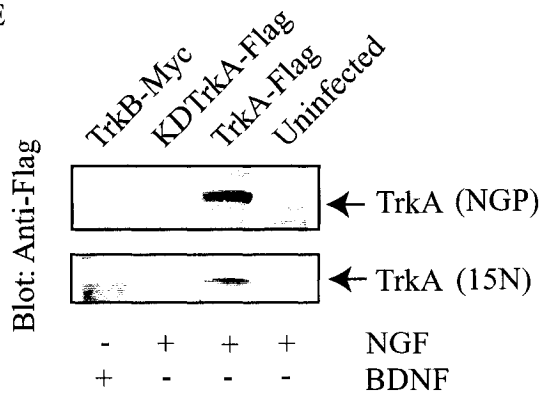
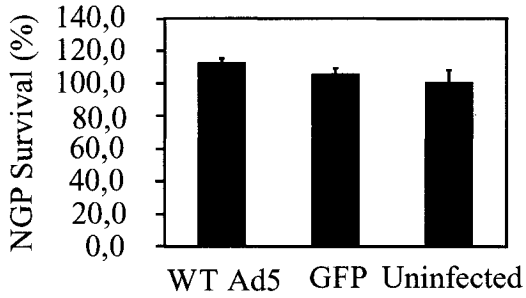


Figure 2.2: Expression of TrkA Ad5 in NGP and 15N neuroblastoma cell lines. (A) Immunostaining for anti-Flag (red) on NGP cells infected for 27hrs with 50 MOI of TrkA-Flag Ad5. Cell nuclei were counterstained with Hoechst (blue) and pictures were taken with a 40X objective. (B) Western blotting with anti-Trk 203 antibody on Trk immunoprecipitated with pan-Trk 203 from NGP cells infected for 36 hrs in absence of neurotrophins with 25 MOI TTA, 50 MOI TrkA or 100 MOI TrkB. (C) Reprobe of (B) using flag antibody. (D) Western blotting with anti-pan Trk 203 antibody on total lysate of NGP cells infected for 36 hrs with either 50 MOI of TrkA, 100 MOI TrkB or 50 MOI LacZ control virus. The neurotrophins were added 5 min prior lysis. The bottom panel was probed for Akt as a loading control. (E) Western Blot for anti-Flag on total lysates obtained from NGP (top) or 15N (bottom) cells infected with 50 MOI of either TrkA, KDTrkA or 100 MOI of TrkB.

either infected with the TrkB Ad5 or co-infected with TrkB Ad5 and Tta Ad5 for the same period of time. The TrkB Ad5, which encodes the myc epitope-tagged TrkB protein under the same promoter as in TrkA Ad5, does not require the presence of Tta to induce TrkB expression in neurons (Atwal et al., 2000). The immunoprecipitated Trk proteins were then visualized by Western blotting using the same pan-Trk antibody. As expected, the uninfected NGP cells did not express any detectable levels of Trk expression. However, TrkA and TrkB expression was detected in TrkA Ad5 and TrkB Ad5-infected NGP cells, respectively (figure 2.2B). In addition, the presence of Tta was dispensable for TrkA and TrkB expression, although in presence of Tta, Trk expression levels were increased. These observations are consistent with previous reports (Atwal et al., 2000). A reprobe of the same blot with an anti-Flag, which recognizes TrkA-Flag but not TrkB-myc, confirmed the ectopic expression of TrkA induced by TrkA Ad5 (figure 2.2C). Similar results were obtained by Western blotting using the 203A antibody of lysates of NGP cells infected with only TrkA Ad5, or the control viruses, TrkB Ad5 and LacZ Ad5, the latter which expresses the beta-galactosidase enzyme under a WT CMV promoter (figure 2.2D). The molecular weight analysis of the bands detected with the pan-Trk antibody revealed that the two major bands observed in TrkA-expressing NGP cell lysates run at 120 and 107 KDa, with a minor band at 86 KDa. In TrkB-expressing cell lysates, two major bands were observed at 124 and 114 KDa and a minor band at 95 KDa. The reprobe of the blot with a Flag antibody, which recognizes the Flag epitope located at the extracellular portion of the TrkA receptor in TrkA Ad5, confirmed these bands were the ectopically-expressed TrkA. The Flag antibody did not efficiently recognize the 120 kDa TrkA, suggesting that the glycosylation of TrkA occurring in the extracellular domain may mask the Flag epitope. Similarly, the various molecular weight bands observed for TrkA and TrkB likely represent different glycosylated forms of the Trk proteins, consistent with previous reports (Hempstead et al., 1992).. The expression of ectopically-expressed TrkA following TrkA Ad5 infection was confirmed with the FLAG antibody in a second neuroblastoma cell line, 15N (figure 2.2E).

A


NGP Survival (%)
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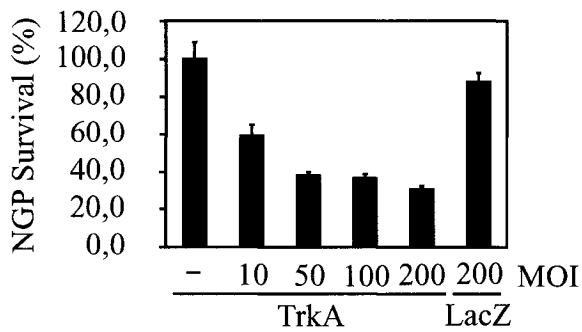
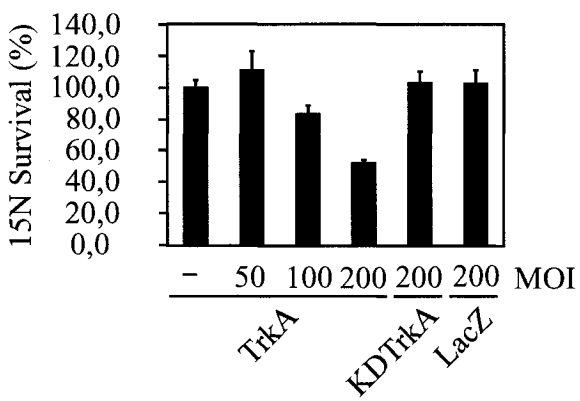
120,0
100,0
80,0
60,0
40,0
20,0
0,0
WT Ad5
GFP
Uninfected

B


NGP Survival (%)
120,0
100,0
80,0
60,0
40,0
20,0
0,0
-
10
50
100
200
200
MOI
TrkA
LacZ

C


15N Survival (%)
140,0
120,0
100,0
80,0
60,0
40,0
20,0
0,0
-
50
100
200
200
200
MOI
TrkA
KDTrkA
LacZ

D

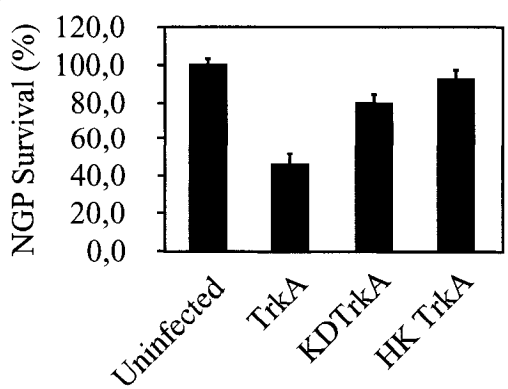
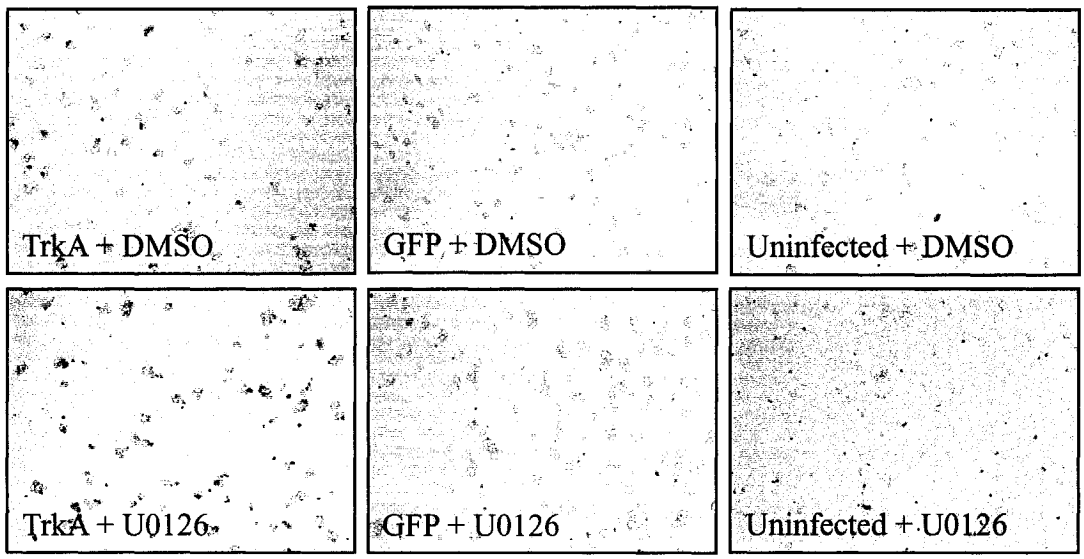

NGP Survival (%)
120,0
100,0
80,0
60,0
40,0
20,0
0,0
Uninfected
TrkA
KDTrkA
HK TrkA

Figure 2.3: TrkA expression induces cell death of both 15N and NGP neuroblastoma cell lines. Survival of NGP (A, B and D) or 15N (C) was assessed 48 hrs post infection by MTT survival assay. Each graphic represents a representative experiment and the error bars correspond to the standard deviation of the mean. A) NGP cells were infected with 50 MOI of GFP or WT Ad5. B) NGP cells infected with increasing MOI of TrkA or 200 MOI of LacZ in presence of NGF (50 ng/ml). C) 15N cells were infected with indicated MOI of TrkA, KDTrkA or LacZ Ad5 in presence of 50ng/ml NGF. D) NGP cells were infected with 50 MOI of TrkA, KDTrkA or heat killed TrkA Ad5 and the cells were treated at the same time with 50 ng/ml of NGF.

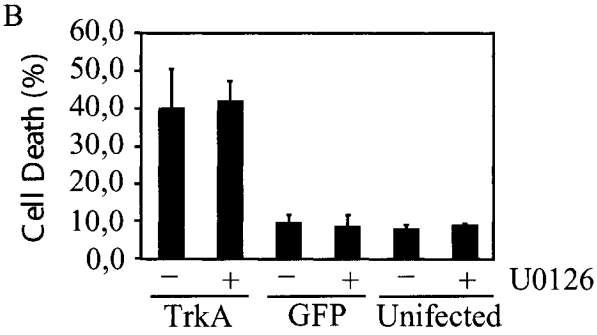
Purified TrkA Ad5 induces cell death in NGP and 15N cells

Preliminary data from Dr. Lynne LeSauteur suggested that infection of neuroblastoma cells with WT contaminated TrkA Ad5 results in the induction of cell death. However, it was not known whether the cell death was due to adenoviral gene expression from the WT virus, to TrkA overexpression, or to a combination of TrkA and WT gene expression. To determine this, we asked (1) if expression of TrkA alone was sufficient to induce cell death, and (2) whether expression of WT Ad5 by itself induced cell death. The survival of NGP cells infected with pure TrkA Ad5, WT Ad5, or control GFP (green fluorescent protein) was assessed by MTT assay three days post-infection, the same time point used by Dr. LeSauteur to assess survival in these cells. Infection of NGP cells with WT Ad5 did not result in decreased cell survival as compared to GFP-infected or uninfected cells (figure 2.3A). In contrast, infection with pure TrkA, but not kinase-inactive TrkA (KDTrkA) decreased the survival of NGF-treated NGP cells (figure 2.3A,B). Similar results were obtained with the 15N cells infected with pure TrkA or KDTrkA (figure 2.3C). The lower amount of cell death observed in 15N cells as compared to NGP cells at equivalent MOIs may be explained by the lower expression levels of TrkA in 15N (figure 2.2D). As a control for contaminants in the viral preparation that might cause cytotoxicity, NGP cells were treated with heat killed (HK) TrkA Ad5. HKTrkA Ad5-infected cells exhibited similar survival to uninfected NGP cells (figure 2.3B). The survival of pure TrkA Ad5-infected NGP cells were decreased by approximately 55% as compared to HKTrkA treated cells (figure 2.3B). TrkA also induced cell death in a dose-dependent manner. NGP cells were infected with various MOIs of TrkA Ad5 or LacZ control virus at the maximal MOI used for TrkA Ad5. LacZ Ad5 infection at 200 MOI resulted in a decrease in cell survival of less than 10% as compared to uninfected cells. The survival of NGP cells was decreased in TrkA Ad5-infected cells in a dose response manner, reaching a maximum of approximately 70% cell death in cells infected with 200 MOI (figure 2.3C). Thus, TrkA, but not kinase-inactive TrkA or WT adenovirus genes, efficiently induces neuroblastoma cell death.

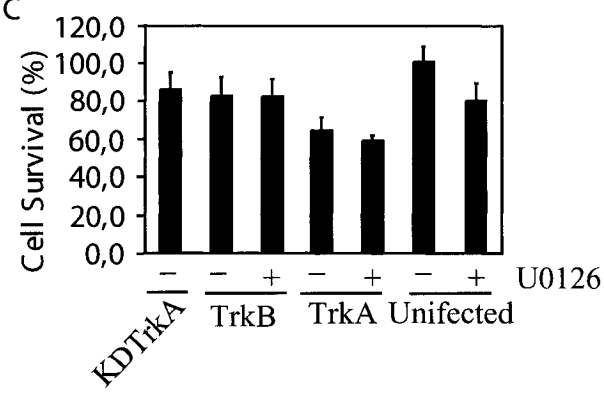
A


TrkA + DMSO
GFP + DMSO
Uninfected + DMSO
TrkA + U0126
GFP + U0126
Uninfected + U0126

B


Cell Death (%)
60,0
50,0
40,0
30,0
20,0
10,0
0,0
− + − + − +
U0126
TrkA
GFP
Unifected

C


Cell Survival (%)
120,0
100,0
80,0
60,0
40,0
20,0
0,0
− − + − + − +
U0126
KDTrkA
TrkB
TrkA
Unifected

D

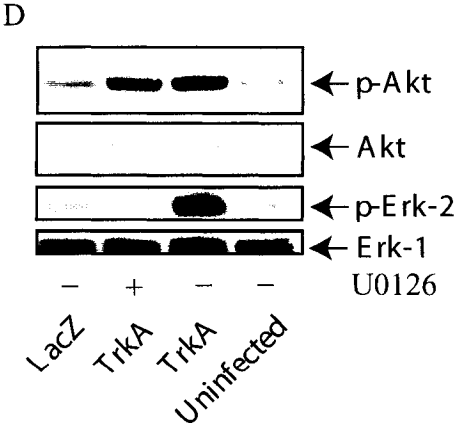

p-Akt
Akt
p-Erk-2
Erk-1
− + − −
U0126
LacZ
TrkA
TrkA
Uninfected

Figure 2.4: Inhibition of ERK does not rescue NGP cells from pure TrkA-induced cell death. (A) DICIII pictures of NGP cells infected with 50MOI of either TrkA or GFP Ad5, in presence of 50 ng/ml of NGF and treated with either the MEK inhibitor U0126 (20 μM) or DMSO for 50 hrs. Cells were stained with trypan blue and pictures were taken. (B) Trypan blue exclusion assay of NGP cells treated as in (A). Cell death corresponds to average of two experiments and the error bar corresponds to standard deviation for these two experiments. (C) One representative MTT survival assay of NGP cells infected for 48 hrs with 50 MOI of TrkA, 50 MOI KD TrkA or 100MOI TrkB and treated with U0126 (+) or DMSO (-). Error bars correspond to the standard deviation of quintaplicate wells. (D) Western Blot of NGP cells infected with either 50 MOI TrkA or LacZ adenovirus for 36 hrs in presence of U0126 (20 μM) or DMSO and treated for 5 min prior to lysis with 50 ng/ml of NGF. Activity of Akt and ERK were determined using phospho-specific antibodies and the membrane was reprobed for total Akt or ERK1 as loading control.

Inhibition of MEK does not suppress TrkA-induced cell death

Unpublished results from our laboratory indicated that inhibition of MEK activity suppressed the cell death-inducing affect of TrkA overexpression in neuroblastoma cells. Since these results were obtained with the WT-contaminated TrkA Ad5, we decided to determine whether TrkA expression in the absence of WT Ad5 co-infection mediates cell death via the MEK/ERK1/2 signaling cascade. We first determined whether TrkA overexpressed via recombinant adenovirus was biochemically functional. Two well-described signal transduction cascades activated by the TrkA receptor in neuronal cells are the Ras-Raf-MEK1/2-ERK1/2 and the PI-3K/Akt pathways (Kaplan and Miller, 2000). Since the activity of ERK1/2 and Akt correlates with their phosphorylation at specific residues, we assessed the activation of these two kinases by Western blotting using phospho-specific antibodies. NGF treatment of NGP cells infected with TrkA Ad5 markedly enhanced the phosphorylation of ERK1/2 and Akt (figure 2.4D). NGF-enhanced ERK1/2 and Akt phosphorylation was not observed in NGP cells infected with control LacZ Ad5, or in uninfected (figure 2.4D). Taken together, these results indicate that the adenovirus-expressed TrkA is biochemically functional.

To inhibit the MEK signaling pathway, we used U0126, a selective pharmacological inhibitor of MEK that we have previously used to suppress MEK activity in sympathetic neurons (Atwal et al., 2000). We assessed the efficacy of this inhibitor by determining the phosphorylation state of the MEK targets ERK1/2, and then the effect of MEK1/2-ERK1/2 inhibition on TrkA-mediated cell death. To confirm that treatment with U0126 inhibits specifically inhibits ERK phosphorylation and not other TrkA signaling cascades, we determined the phosphorylation state of Akt in neuroblastoma cells treated with this inhibitor. Western blotting was performed with phospho-specific anti-ERK and anti-Akt antibodies on lysates of NGP cells that were infected with pure TrkA Ad5 or the LacZ control virus for 36 hrs in presence of 20 μM of U0126 or vehicle, and treated for 5 min with 50 ng/ml NGF just prior to cell lysis. As expected, the phosphorylation levels of Akt and ERK1/2 were increased in NGF-treated NGP cells that were infected with TrkA Ad5, but not in the uninfected or LacZ Ad5-infected cells (figure 2.4D). Treatment with U0126, but not the drug vehicle, was sufficient to prevent NGF-mediated ERK1/2 phosphorylation without having any effects

on Akt phosphorylation (figure 2.4D). To assess whether MEK inhibition suppressed TrkA-induced cell death, NGP cells infected with adenoviruses encoding pure TrkA, KDTrkA, TrkB, or GFP were treated with U0126 or DMSO vehicle. The survival of these cells was then assessed by MTT (which assesses mitochondrial activity as a measurement of cell proliferation and survival) or Trypan Blue exclusion assay (which assesses both apoptotic and non-apoptotic cell death) 48 hrs post-infection. TrkA Ad5 infection resulted in 40% cell death, which was not reduced by treatment with U0126 (figure 2.4A-C). TrkB, KDTrkB, and GFP infection did not significantly reduce cell survival (figure 2.4A-C). Therefore, TrkA-induced NGP cell death does not require the activity of MEK.

TrkA does not induce cell death via the rapid activation of p38MAPK or JNK

Two cell death signaling cascades that have been identified to play roles in the death of neural cell types are MEK3/6-p38MAPK in TrkA-overexpressing PC12 cells (Yan et al., 2002) and TrkC-expressing medulloblastoma cells (Kim et al., 1999), and the MKK4/7-JNK pathway in sympathetic neurons (Miller and Kaplan, 2001). Therefore we sought to determine these two pro-cell death pathways are implicated in TrkA-mediated cell death of neuroblastoma cell. Since the phosphorylation of p38MAPK and JNK at specific residues is required for their enzymatic activity, we determined by Western blotting the phosphorylation status of these two kinases using phospho-specific antibodies. To determine whether TrkA induces the activation of the MEK3/6-p38MAPK pathway, TrkA or LacZ-infected NGP cells were treated with NGF for 5 minutes prior to lysis. This treatment time has been reported by the group of Schor to be sufficient to induce MEK3/6 phosphorylation in PC12 cells overexpressing TrkA (Yan et al., 2002). Western blotting of these lysates showed no increase in p38MAPK phosphorylation in TrkA-infected NGP cells as compared to LacZ Ad5-infected or uninfected NGP cells (figure 2.5A). However, it must be pointed out that the levels of p38MAPK were very low in the NGP cell line. We conclude that TrkA does not induce cell death by the rapid activation of the MEK3/6-p38MAPK cascade.

We next asked whether JNK was activated by TrkA overexpression in NGP cells. NGP cells were infected with TrkA Ad5 or LacZ Ad5, treated with NGF for 10

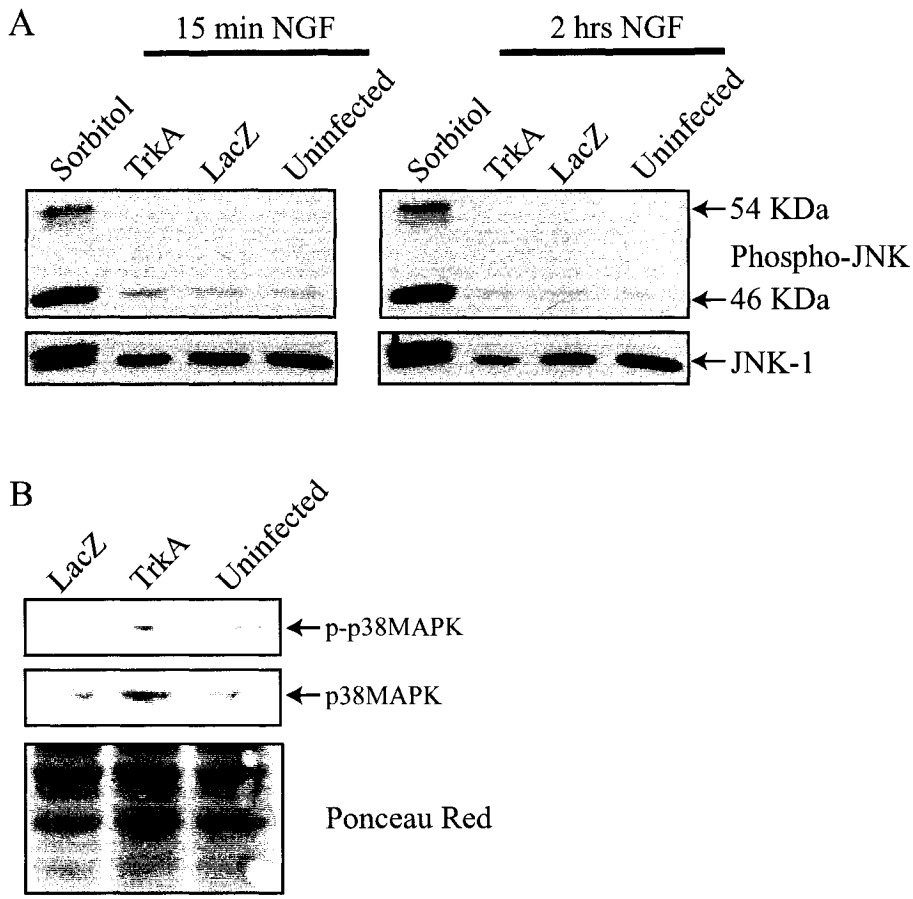


Figure 2.5: TrkA expression does not lead to the activation of JNK and p38MAPK pathways. Western Blots for phospho-JNK and phospho-p38MAPK. NGP cells infected with 50 MOI TrkA or LacZ Ad5 for 36 hrs were treated with 50 ng/ml of NGF for 5 min (B), 15 min or 2hrs (A) prior lysis. (A) Uninfected NGP cell treated with 0,5 mol of sorbitol was used as positive control for phospho-JNK. Bottom panels are reprobed for JNK-1 that was used as loading control. (B, middle) Membrane was reprobed with anti-p38MAPK as loading control. As an additional loading control, Ponceau red of the membrane corresponding to the molecular weight of p38MAPK is presented (lower panel).

minutes or 2 hours, and phosphorylation assessed. As a positive control for JNK phosphorylation, uninfected NGP cells were treated with 0.5 M sorbitol for 15 minutes, which induces the phosphorylation of JNK (figure 2.5B). While sorbitol induced JNK phosphorylation, TrkA overexpression did not at either time point. Taken together, these experiments suggest that TrkA activation does not induce the rapid activation of JNK or p38MAPK.

TrkA induced activation of pRB and upregulation of $p21^{WAF}$

Another possible mechanism by which TrkA could induce cell death is by producing conflicting cell cycle signals that might "confuse" the neuroblastoma cells, and subsequently cause the induction of apoptosis. The hypothesis is that TrkA will inhibit the cell cycle in cells that are constitutively cycling due to the high levels of N-myc expression, producing conflicting signals that subsequently cause the activation of apoptotic genes such as p53. To determine whether TrkA induced the expression of anti-mitotic proteins, we analysed the expression levels or activity of two known cell cycle repressors, the retinoblastoma protein (pRB) and the cyclin/Cdk (cyclin-dependent kinase) inhibitor $p21^{WAF}$. The activity of pRB is regulated by phosphorylation; hyperphosphorylated pRB is inactive, while hypophosphorylated pRB is active and prevents cell cycle progression. Since hyperphosphorylated pRB migrates at a higher molecular weight than hypophosphorylated pRB on SDS-PAGE, the phosphorylation levels of pRB can be determined by Western blotting either using a phospho-specific antibody which recognizes the hyperphosphorylated form, or by using a pan-pRB antibody which will detect the shift in the apparent pRB molecular weight as a measurement for pRB phosphorylation levels. NGP cells were infected with either TrkA Ad5 or LacZ Ad5 for 36 hrs in the presence of NGF. Using a phospho-specific pRB antibody, pRB was found to be phosphorylated in both uninfected and LacZ infected NGP cells, which was expected as these cells were proliferating (figure 2.6A). In contrast, phospho-pRB levels were dramatically reduced in TrkA-expressing cells (figure 2.6A). We did not perform this experiment in the absence of NGF because once overexpressed, TrkA can auto-activate in an NGF-independent manner (Hempstead et al., 1992) leading to the activation of TrkA signalling cascades. In addition, in the

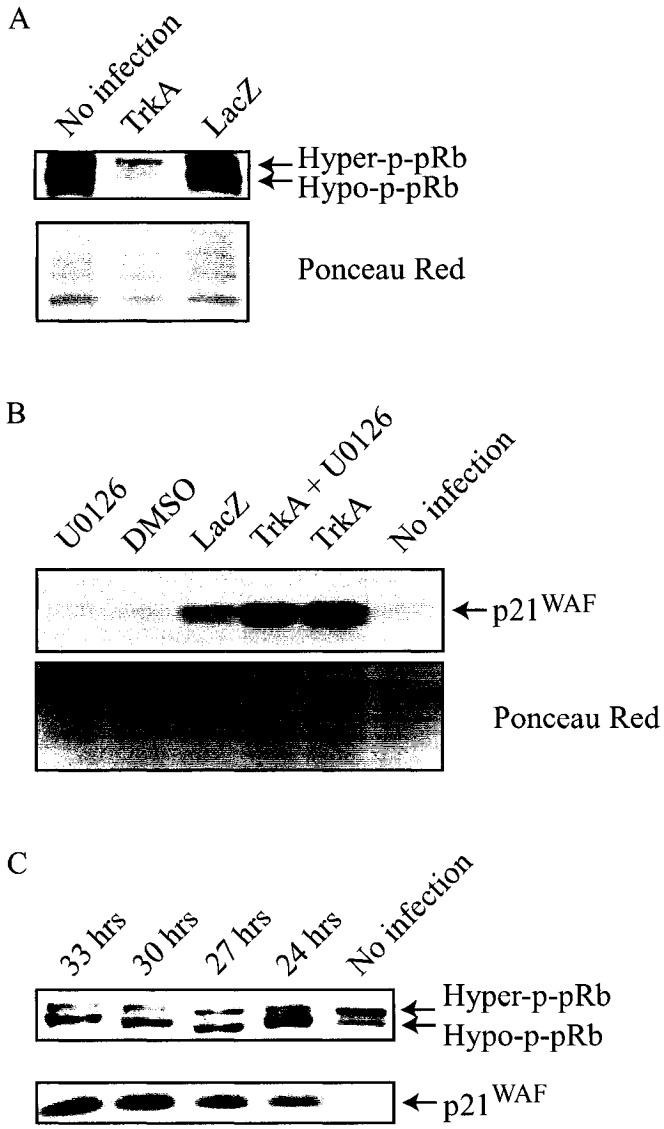


Figure 2.6: The cell cycle repressors, pRB and $p21^{WAF}$ are activated and up-regulated, respectively, following TrkA expression. (A) Western blot for phospho-pRB on lysates of NGP cells infected with 50 MOI of either TrkA or LacZ Ad5 for 36 hrs in presence of 50 ng/ml of NGF. As a loading control, the lower panel shows the Ponceau red of the area corresponding to pRB. (B) Western blot for $p21^{WAF}$ on lysates of NGP cells infected as in (A) and treated with U0126 (20 uM) or DMSO. The bottom panel shows the Ponceau red used as loading control. (C) Western blot for pan-pRB and $p21^{WAF}$ of NGP cells infected with TrkA Ad5 for indicated times in presence of NGF.

absence of NGF, overexpression of TrkA will kill neuroblastoma cells, although at a slower rate than in NGF (data not shown). To determine whether this reduction of phospho-pRB levels was attributable to reductions of pRB protein, and to determine when pRB became dephosphorylated following TrkA expression, NGP cells were infected with 50 MOI TrkA for 24 to 33 hrs in presence of NGF, and pRB protein levels assessed by Western blotting using anti-pRB. While the levels of pRB protein were similar in TrkA-expressing and uninfected NGP cells, the band intensity for the hypophosphorylated form of pRB was more intense in uninfected cells than in TrkA-expressing cells (figure 2.6C). This result suggests that TrkA expression causes a decrease in pRB phosphorylation rather than an overall decrease in pRB expression. In addition, the unphosphorylated form of pRB was detectable by 24 hrs post-TrkA infection (upper band of figure 2.6C). Therefore, expression of TrkA results in the dephosphorylation of pRB protein in NGP cells, likely resulting in the activation of pRB. However, these experiments do not show how TrkA induces this change in pRB phosphorylation.

We also asked whether expression of another cell cycle inhibitor, $p21^{WAF}$, was induced by TrkA in NGP cells. Since $p21^{WAF}$ activity is regulated by protein expression levels, we performed Western blotting using a $p21^{WAF}$ antibody to determine the levels of $p21^{WAF}$ in NGP cells. As expected, uninfected NGP cells expressed barely detectable $p21^{WAF}$ protein (figure 2.6B). In contrast, TrkA Ad5-infected NGP cells exhibited high $p21^{WAF}$ levels (figure 2.6B), both in comparison to uninfected and LacZ-infected cells. We then determined the temporal pattern of TrkA induced $p21^{WAF}$ expression in NGP cells infected with TrkA Ad5 for 24hrs to 33 hrs in presence of NGF. $p21^{WAF}$ expression was detectable by 24 hrs post-infection, and increased to reach maximal expression by 30 hrs (figure 2.6C, lower panel). Taken together, these experiments suggest that TrkA induces cell cycle arrest by activating at least two cell cycle repressors, pRB and $p21^{WAF}$.

TrkA up-regulates p53 protein levels

Since $p21^{WAF}$ expression can be induced by the tumour suppressor protein p53, and since p53 plays an important role in cell death induction, we asked whether TrkA

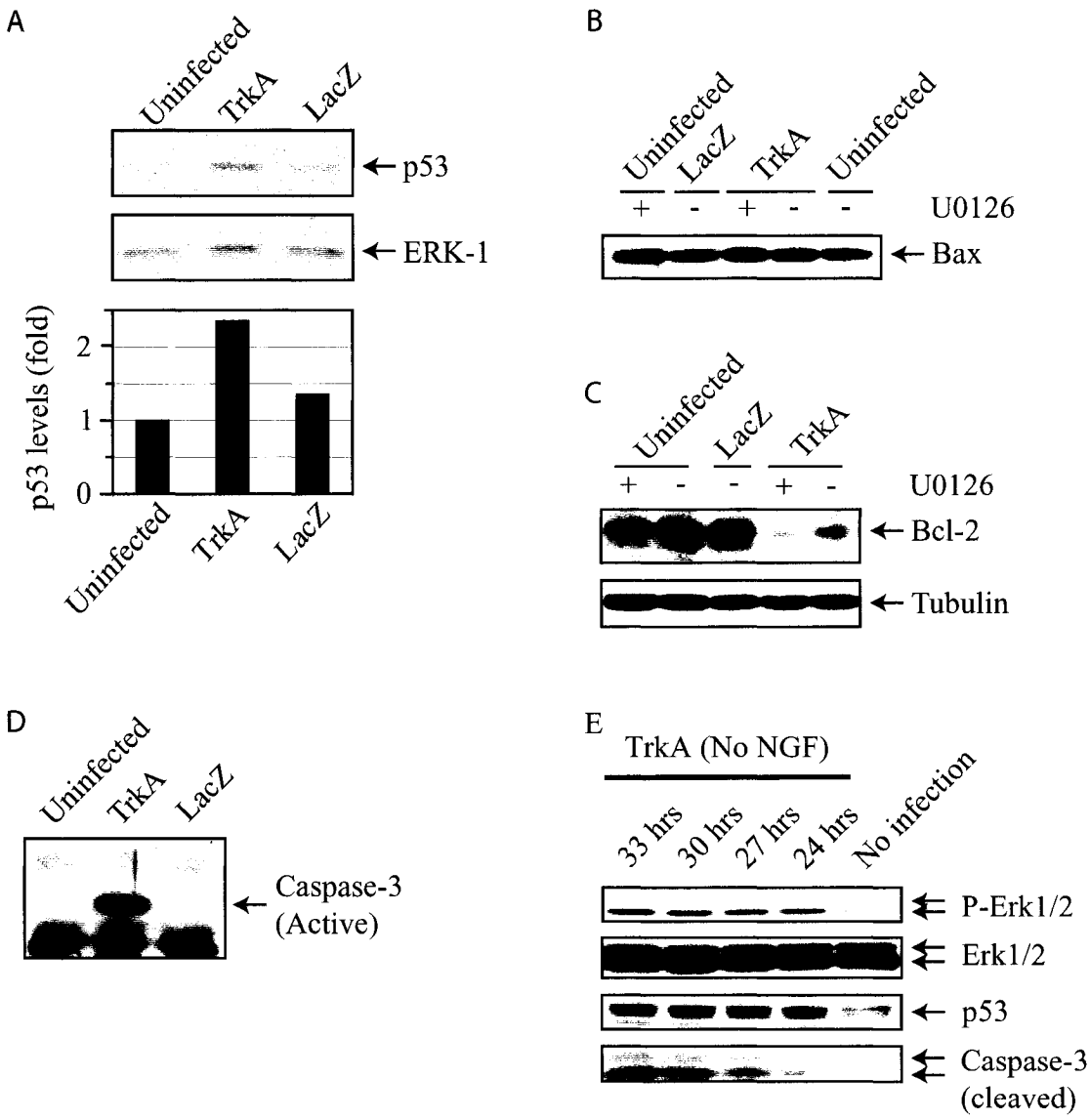

A
Uninfected
TrkA
LacZ
p53
ERK-1
p53 levels (fold)
2
1
0
Uninfected
TrkA
LacZ
B
Uninfected
LacZ
TrkA
Uninfected
+ - + - -
U0126
Bax
C
Uninfected
LacZ
TrkA
+ - - + -
U0126
Bcl-2
Tubulin
D
Uninfected
TrkA
LacZ
Caspase-3
(Active)
E
TrkA (No NGF)
33 hrs
30 hrs
27 hrs
24 hrs
No infection
P-Erk1/2
Erk1/2
p53
Caspase-3
(cleaved)
F
TrkA + NGF
35 31.5 27 25
No infection
(Hrs post-infection)
PARP(cleaved)

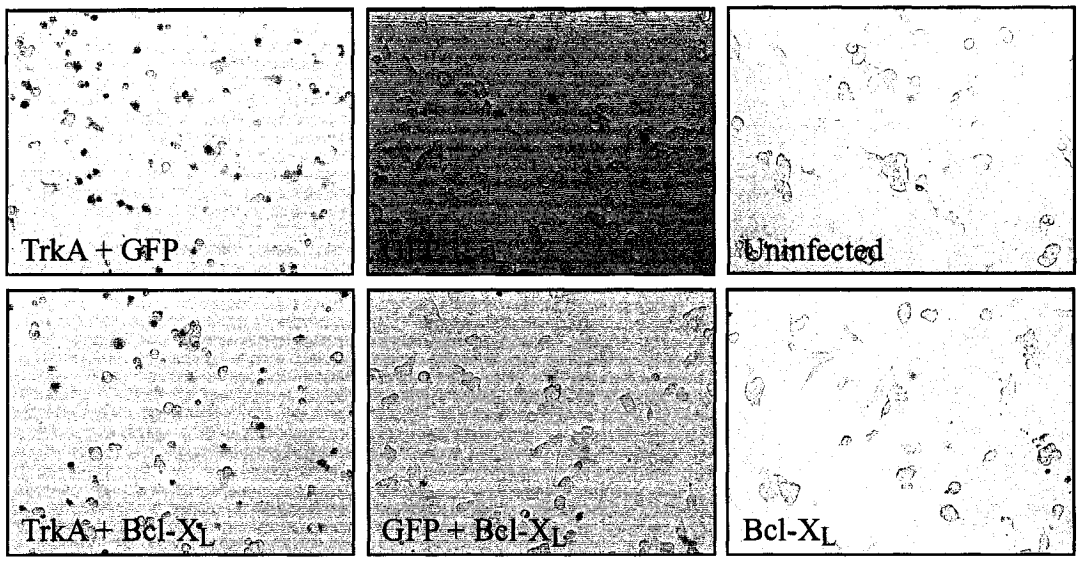
Figure 2.7: TrkA induces the up-regulation of pro-apoptotic proteins and the decrease of the anti-apoptotic protein, Bcl-2. The protein levels of p53 (A), Bax (B), Bcl-2 (C), and active-caspase-3 (D) were determined by Western blot on lysates of NGP cells infected with 50 MOI of TrkA or LacZ Ad5 in presence of NGF (50 ng/ml) for 36 hrs. (A) Levels of p53 (top panel) were normalized to the levels of ERK-1 (middle panel). This normalized value was divided by the p53 normalized value for uninfected cells to determine the increase (in fold) of p53 in infected cells (bottom panel). (B) NGP cells were infected as described above, and treated with U0126 (20 μM) or DMSO. (C) NGP cells were treated as in (B) and levels of Bcl-2 were determined (top panel). The second panel shows the levels of tubulin, which was used as a loading control. (D) Levels of cleaved (active) caspase-3 in NGP cells infected with either TrkA Ad5 or LacZ Ad5. Levels of ERK phosphorylation (E), and the protein levels of p53 (E), cleaved caspase-3 (E) and cleaved PARP (F) were determined at indicated time points following TrkA Ad5 infection in absence (E) or presence (F) of NGF.

expression in NGP cells increased p53 protein levels. This was determined by Western blotting using a p53 antibody of lysates of NGP cells that were infected with TrkA or LacZ Ad5. p53 protein levels were two-fold higher in TrkA-expressing NGP cells than in LacZ-expressing cells (figure 2.7A). Similar results were obtained in multiple experiments (figure 2.7E). In the absence of NGF, this increase in p53 was detectable by 24 hrs post-infection, and increased with time to reach a maximal level by 30 hrs post infection (figure 2.7E). This result is not surprising, as over-expressed TrkA can auto-phosphorylate in the absence of NGF (Hempstead et al., 1992). In support of the auto-activation of TrkA in this experiment, we observed that ERK1/2 MAP kinase was phosphorylated in cell lysates of TrkA-expressing NGP cells (figure 2.7E). These results suggest that TrkA may induce the death of neuroblastoma cells in part via increases in the levels of the p53 apoptotic protein.

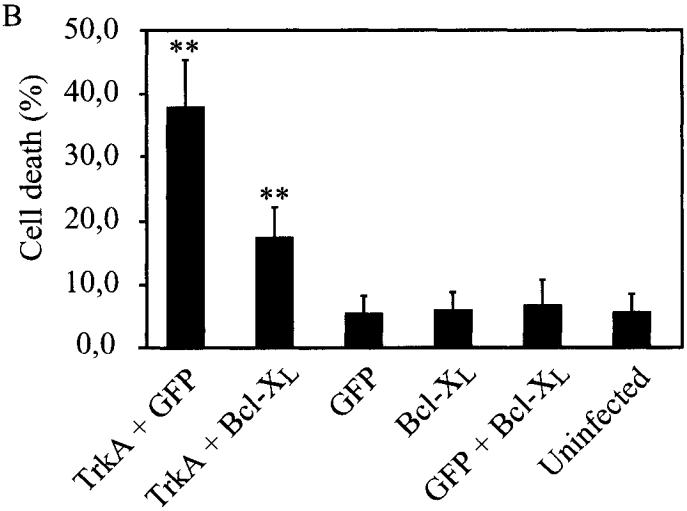
TrkA induces a down-regulation of Bcl-2 protein levels

Increases in p53 protein levels often results in the transcription of pro-apoptotic genes including the pro-apoptotic Bcl-2 family member protein, Bax (Miyashita and Reed, 1995). Therefore we asked whether TrkA expression resulted in the up-regulation of Bax protein levels. Bax levels, however, were not altered by TrkA expression, as determined by Western blotting with anti-Bax (Figure 2.7B). Since the balance between pro- and anti-apoptotic Bcl-2 family members can regulate apoptosis, we asked whether TrkA expression altered the levels of other BH3 domain-containing proteins such as Bcl-2. Bcl-2 levels were almost completely suppressed by TrkA expression, as determined by Western blotting using with anti-Bcl-2 (figure 2.7C). The dramatic decrease in Bcl-2 levels may account for TrkA's cell death-inducing activity. If TrkA causes cell death in part by decreasing Bcl-2 levels, than reconstituting Bcl-2 family member expression should prevent TrkA-induced cell death. Since we did not have an adenovirus capable of expressing Bcl-2 in neuroblastoma cells, we used an adenovirus expressing the anti-apoptotic Bcl-2 family member Bcl-X_L. Using this virus, Bcl-X_L was expressed at approximately 30-50% higher levels than in LacZ-infected or uninfected cells, as determined by Western blotting with anti-Bcl-X_L (figure 2.8C). To determine whether Bcl-X_L overexpression would prevent TrkA-induced cell death, NGP cells were co-

A


TrkA + GFP
Uninfected
TrkA + Bcl-XL
GFP + Bcl-XL
Bcl-XL

B


Cell death (%)
50,0
40,0
30,0
20,0
10,0
0,0
**
**
TrkA + GFP
TrkA + Bcl-XL
GFP
Bcl-XL
GFP + Bcl-XL
Uninfected

C

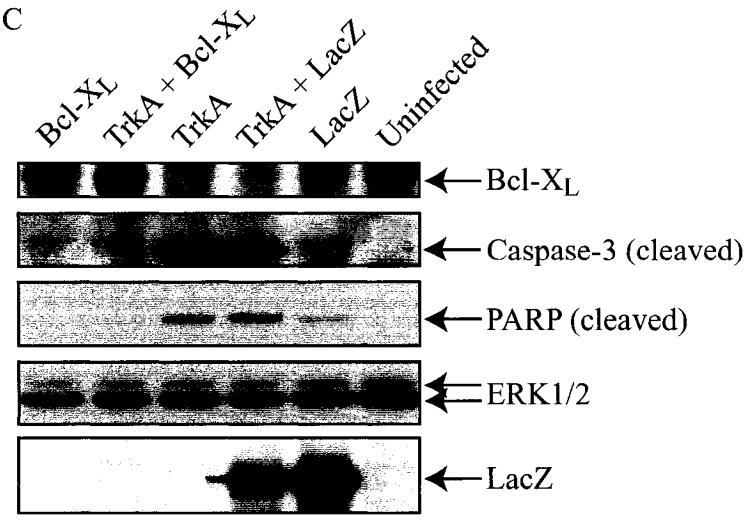

Bcl-XL
TrkA + Bcl-XL
TrkA
TrkA + LacZ
LacZ
Uninfected
Bcl-XL
Caspase-3 (cleaved)
PARP (cleaved)
ERK1/2
LacZ

Figure 2.8: Over-expression of Bcl-X_L protects NGP cells from TrkA-induced cell death and prevents caspase-3 and PARP cleavages. (A-B) NGP cells were infected with 50 MOI of TrkA, GFP or Bcl-X_L Ad5, as indicated, in presence of 50 ng/ml of NGF for 50-52 hrs. Cells were stained with Trypan blue for 10 min and pictures were taken (A). The number of dead cells was counted for each conditions divided and by the total number of cells per field. The average of four independent experiments is represented in (B). The error bar corresponds to the standard deviation of the mean among these four experiments. (** = $p \leq 0,01$; t-Test). (C) Western blotting for Bcl-X_L, active-caspse-3, cleave PARP and LacZ on lysates of NGP cells treated as in A and B for 36 hrs. For loading control, the membrane was also probed for anti-ERK.

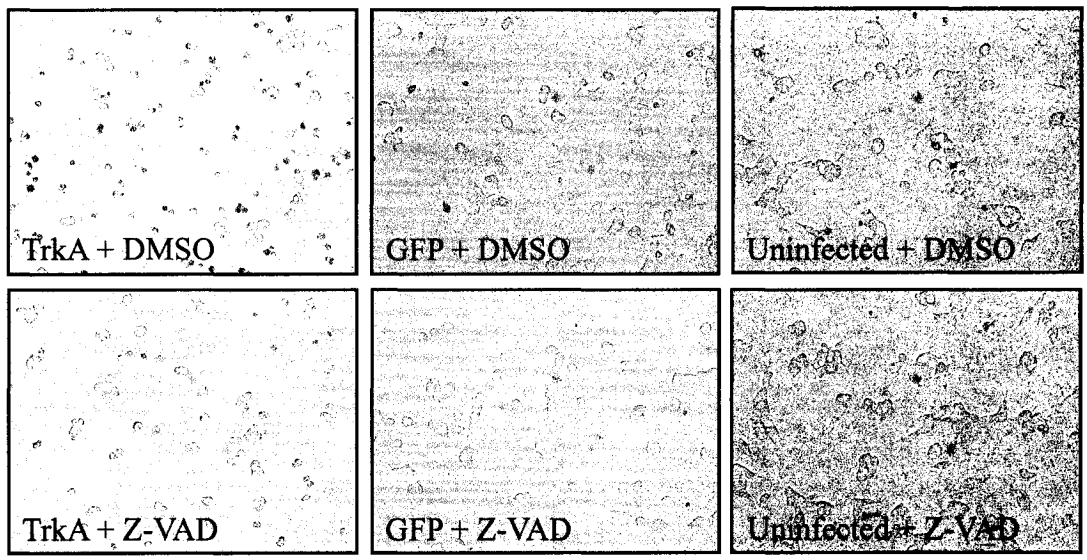
infected for 48 hrs with TrkA and Bcl-X_L adenoviruses, or control GFP adenovirus, and cell death assessed by Trypan blue exclusion assay. Bcl-X_L overexpression reduced TrkA-induced cell death by 54,8 ± 6,3 % (n = 4 independent experiments, $p \leq 0.01$, t-Test), while GFP expression had no effect (figure 2.8A-B). Bcl-X_L was expressed in cells infected with TrkA, as determined by Western blotting with anti-Bcl-X_L (figure 2.8C). These results suggest that TrkA induces cell death by decreasing the levels of the anti-apoptotic Bcl-2 protein.

TrkA expression induces activation of caspase-3

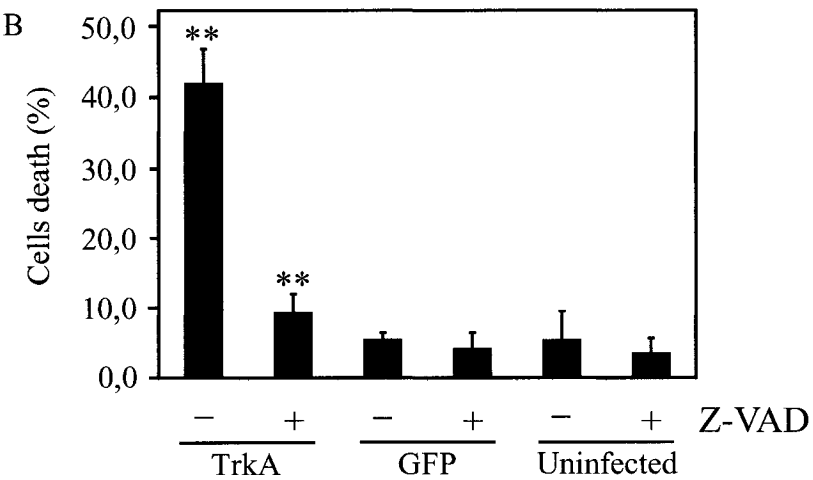
A decrease in Bcl-2 levels leads to the release of mitochondrial cytochrome c, the activation of caspase-9, and the subsequent cleavage and activation of the effector caspase, caspase-3 (see chapter I). We therefore examined whether TrkA induced cell death in a caspase-dependent manner. Caspase-3 activity was assessed in two ways; by the appearance of cleaved caspase-3 and by the appearance of the cleaved form of the caspase-3 target poly(ADP-Ribose)polymerase (PARP) by Western blotting using antibodies that specifically recognize the cleaved forms of these proteins. NGP cells were infected with TrkA Ad5 or LacZ Ad5 for 36 hrs, lysed and the levels of cleaved caspase-3 and PARP determined. Caspase-3 was activated in TrkA expressing, but not LacZ-expressing NGP cells (figure 2.7D). Active caspase-3 was first apparent at 24 hrs post-TrkA infection and increased to reach a plateau at 30 hrs post-infection (figure 2.7E). Consistent with the increase in caspase-3 activation following TrkA expression, we observed an increase in the cleaved form of PARP in NGP cells infected with TrkA Ad5, but not with control virus (figure 2.7F). This increase in cleaved PARP levels in TrkA-infected cells was first observed at 25 hrs post-infection, and increased with time (figure 2.7F).

To determine whether the activity of caspases was required for TrkA-induced cell death, TrkA or control virus-infected NGP cells were treated with the broad-spectrum caspase inhibitor Z-VAD-fmk. Cell death was assayed by Trypan blue exclusion assay two days post-treatment, and the ability of Z-VAD-fmk to prevent caspase-3 activation was confirmed by Western blotting. As shown in figure 2.9A treatment of TrkA-infected NGP cells with 50 ng/ml of Z-VAD-fmk was sufficient to

A


TrkA + DMSO
GFP + DMSO
Uninfected + DMSO
TrkA + Z-VAD
GFP + Z-VAD
Uninfected + Z-VAD

B


Cells death (%)
50,0
40,0
30,0
20,0
10,0
0,0
**
**
− + − + − +
Z-VAD
TrkA
GFP
Uninfected

C

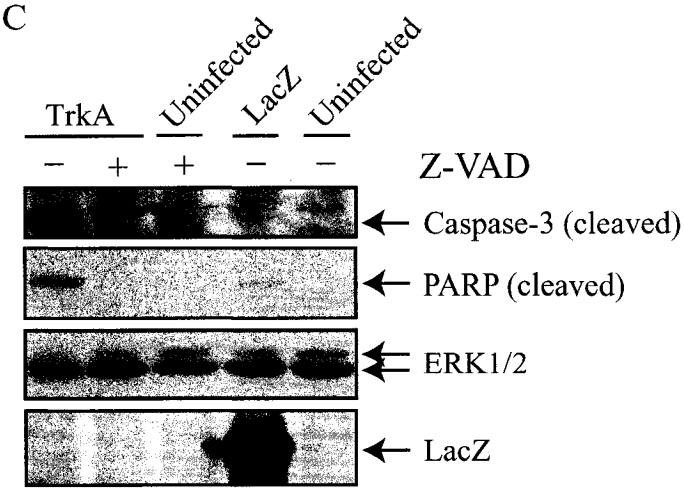

TrkA
Uninfected
LacZ
Uninfected
− + + − −
Z-VAD
Caspase-3 (cleaved)
PARP (cleaved)
ERK1/2
LacZ

Figure 2.9: Broad caspase inhibitor rescues NGP cells from TrkA-induced cell death and prevents caspase-3 and PARP cleavage. NGP cells were infected with 50 MOI of either TrkA or GFP Ad5 for 50-52 hrs in presence of 50 ng/ml of NGF and treated with either ZVAD-fmk (100 nM) or DMSO (A-B). Cell survival was determined using trypan blue staining (A). The number of dead cells was counted for each condition and divided by the total number of cells per field. The average for three independent experiments is represented in (B). The error bar corresponds to the standard deviation among these three experiments. (** = $p \leq 0.01$; ANOVA). (C) Western blot for active-caspse-3, cleaved PARP and LacZ on lysates of NGP cells treated as in A and B for 36 hrs. For loading control, the membrane was also probed for anti-ERK.

prevent caspase-3 activation and PARP cleavage as measured 36 hrs post-treatment. Consistent with this observation, treatment of TrkA infected cells with ZVAD-fmk decreased TrkA-induced cell death by 78,0 ± 5.5% (n=3, $p \leq 0.01$, ANOVA) (figure 2.9B-C). Treatment of GFP Ad5 infected or uninfected cells with ZVAD-fmk did not alter their survival (figure 11B-C). This result suggests that TrkA induces cell death in a caspase-dependent manner.

To determine whether the protective effect of overexpression of Bcl-X_L occurred upstream of caspase-3, we determined the levels of active caspase-3 and cleaved PARP in cells co-infected with TrkA and LacZ Ad5 or TrkA and Bcl-X_L Ad5. As shown in figure 2.8C, expression of Bcl-X_L, but not GFP, prevented caspase-3 activation and cleavage of PARP. Taken together, these data suggest that TrkA induces apoptosis of the NGP neuroblastoma cell line by increasing p53 protein levels and decreasing Bcl-2 levels, which in turn increases caspase-3 activity and apoptosis.

2.3 Material and Methods:

Cell culture

The human neuroblastoma cell lines Lan-1-15N (15N) (Ciccarone et al., 1989) and NGP (Brodeur et al., 1977) cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (media and supplements were purchased from BioWhittaker). The human kidney HEK 293 and the HEK 293 expressing the transactivator tTa (293 tTa) cells were obtained from Dr. B. Massie and cultured in DMEM (BioWhittaker) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. These four cell lines were passaged using trypsin and grown at 37 ˚C in an atmosphere containing 5% CO_2. The neuroblastoma cells were plated in rat tail collagen coated flasks for biochemistry assays, or on poly-D-Lysine (Sigma, Saint Louis, MI) coated flasks for immunohistochemistry and survival assays.

Neurotrophins and Antibodies:

Nerve growth factor (NGF) was obtained from Cedarlane (Hornby, Ontario) and the brain-derived neurotrophin factor (BDNF) was purchased from PeproTech (Rocky Hill, NJ) and used at 50 ng/ml and 100 ng/ml respectively, for duration of these studies. Anti-pan Trk 203 antibody (raised in rabbit), which was generated against the carboxy-terminal 14 amino acid of the human Trk receptor has previously been described (Hempstead et al., 1992). The anti-phospho tyrosine 4G10 monoclonal antibody was obtained from AEGERA (Iles-des-Soeurs, Québec). Anti-phospho-MAPK specific for phosphorylated residues 183^{Thr} and 185^{Tyr} and the anti-phorpho-p38 MAPK (both raised in rabbit) were bought from Promega (Madison, WI). Rabbit anti-ERK-1, anti-JNK-1, anti-Bcl-2, anti-Bcl-X_L and mouse anti-p53 (DO-1) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-active JNK (rabbit) was bought from BioSource (Camarillo, CA). The antibodies against anti-phospho Akt recognizing phosphorylated serine 473, anti-pan Akt, anti-phospho-Rb specific for phospho-serine 780, anti-cleaved caspase-3 and anti-cleaved-PARP were obtained from Cell Signaling (Bervely, MA). Upstate Biotechnology (Lake placid, NY) was the supplier for anti-$p21^{WAF}$ and anti-Bax. The anti-pan pRB antibody was purchased from PharMingen (Mississauga, ON). The anti-Flag® M2 and anti-tubulin antibodies were obtained from Sigma. These primary antibodies were revealed by Western blotting using goat secondary antibodies anti-mouse (BioRad, Hercules, CA) or anti-rabbit (Chemicon International, Temecula, CA) coupled to HRP or by immunostaining using the secondary antibodies anti-mouse-FITC and anti-rabbit-Cy3 (Jackson ImmunoResearch Laboratories).

Adenoviruses:

Adenovirus encoding for human wild type TrkA (TrkA Ad5) under the tTa-inducible cytomegalovirus (CMV) promoter (tTa-CMV) was previously generated by Josée Wong from Dr. David Kaplan's laboratory. Briefly, the wild type human TrkA cDNA tagged at its extracellular domain with the Flag epitope was excised from pKSTrkA vector obtained from M. Ashcroft and inserted into the pAdTR5F adenovirus vector (named pAdTR5F-TrkA). The pAdTR5F-TrkA was transfected in HEK 293 cells to obtain

adenoviral particles expressing TrkA-Flag. Viral particles were amplified in HEK293 cells and purified on cesium chloride gradients (see below), as previously described (Massie et al., 1995; Mazzoni et al., 1999). Viral titer was determined using the tissue culture infectious dose 50 ($TCID_{50}$) method (see below). TrkB Ad5, which express TrkB tagged with Myc at its extracellular terminus under the tTa-CMV promoter, was generated by Dr. J. Atwal (Atwal et al., 2000). Bcl-X_L Ad5, LacZ Ad5 or GFP Ad5, which express CMV promoter driven Bcl-X_L, beta-galactosidase and GFP, respectively, were obtained from AEGERA.

Amplification and $CsCl_2$ purification

TrkA Ad5 was amplified in HEK 293 cells by successive rounds of infection: infected HEK 293 cells were collected, lysed by 2 cycles of freeze (-80 °C) –thaw (37 °C), and used to infect 65% to 75% confluent HEK 293 cells. For the final round of infection, from 12, 500 cm^2 dishes (Corning Incorporated, Corning, NY) infected HEK 293 cells were collected, spun down and resuspended in a total volume of 11-12 ml of media. The cells were lysed by 4 cycles of freeze-thaw and centrifuged at 3000 RPM to remove large cellular debris. The supernatant containing TrkA Ad5 virions was purified on two successive cesium-chloride (EM Science, Gibbstown, NJ) gradients. TrkA Ad5 isolated from the second gradient was dialysed three times in a dialysis buffer (10mM Tris pH 7.4 (Fisher, Fair Lawn, NJ), 1mM $MgCl_2$ (EM Science) and 10% glycerol (Fisher)) at 4 C (final dilution of $1:10^9$), aliquoted and stored at -80C.

Plaque assay

HEK 293 cells, plated in 60 mm dishes, were infected overnight with serial 10-fold dilutions of cesium chloride purified TrkA-Ad5. The media was then replaced with media containing 1% of SeaPlaque® agarose. Cells were fed every 3-4 days by adding fresh agarose-containing media. At 10 days post-infection plaques were visible. Thirty of these plaques were isolated and eluted in 1 ml of media overnight at 37 °C, and 5% CO_2. One hundred µl of these eluted viruses were used to infect HEK 293 tTa cells plated in 24 well plates. Two days post infection, cells were harvested and TrkA expression was determined by Western Blotting.

Titer determination by $TCID_{50}$ plaque assay

TrkA Ad5 titer was determined using the $TCID_{50}$ assay (Quantum Biotechnologies). Briefly, HEK 293 cells plated in 96 wells plate at a density of 10^4 cells per well were infected with TrkA diluted ten-fold serially. Ten days post-infection, the ratio of positive (wells containing infected HEK 293 cells) to negative wells (wells with no infected cells) was calculated for each viral dilution. These ratios were added and used as the value of S in equation 1*. The TCID50 value was converted into *plaque forming unit per milliliter* by subtracting the constant 0.7 to constant 1 of equation 1.

Equation 1*: $T_{pfu/ml.} = 10^{(1 + d(S - 0.5) - 0.7)}$

d = Log 10 of the dilution (= 1 for a 10 fold dilution)

S = the sum of ratio (ratio of positive well for each viral dilution)

T = is expressed in plaque forming unit per milliliters (pfu/ml)

PCR

The presence of E1A and E1B genes was determined by PCR using the following forward (FWD) and reverse (REV) primers. E1A FWD: 5'-ATC TGC CAC GGA GGT GTT ATT-3', E1A REV: 5'-CGG TAC AAG GTT TGG CAT AGA-3', E1B FWD: 5'-GCT GAA GAC CAT TCA CGT AGC C-3', and E1B REV: 5'-CAG TAC CTC AAT CTG TAT CTT CAT CGC-3'. The E1A and E1B sequences were amplified by denaturing the viral DNA at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and elongation at 72 °C for 1 min. The PCR products were run on a 1.5% agarose gel.

Infections:

Cells at 70-80% confluency were trypsinised, counted on a hemocytometer and plated at similar density in flasks coated with either 4% rat tail collagen (for biochemistry assays) or poly-D-lysine (survival assays or immuohistochemistry) and allowed to settle for 18 to 24 hrs prior treatment. Cells were infected or co-infected with 50 multiplicity of

infection (MOI) TrkA Ad5, 50 MOI kinase dead TrkA Ad5, 100 MOI TrkB Ad5, 50 MOI LacZ Ad5, 50 MOI GFP Ad5, or 50 MOI Bcl-X_L Ad5, unless otherwise indicated. Neurotrophins were added at time of infection for time course experiments and survival assays or acutely added (5 to 15 min) at 36 hrs for biochemistry assays. The pharmaceutical drugs, U0126 MEK inhibitor (Promega) and z-VAD caspase inhibitor (Calbiochem), used at 20 μM and 100nM respectively, were also added at the time of infection.

Biochemistry assays:

Immunoprecipitation: NGP cells were infected for 36 hrs, then treated with 50 ng/ml of NGF for 5 min at 37 °C prior to lysis with NP-40 lysis buffer (TBS: 39.8 mM Tris HCl, 274 mM NaCl, pH 7.4, supplemented with 1% NP-40 (EM Science), 10% glycerol (Fisher), 500 μM sodium vanadate (Fisher) and protease inhibitor cocktail (Roche, Mannhein, Germany)). Cell lysates were equalized using BCA protein assay kit (Pierce, Brockville, ON) and Trk proteins were immunoprecipitated from 90 μg of protein with the anti-pan Trk 203 antibody. For the immunoprecipitation, cell lysates were incubated with pan-Trk 203 antibody for 2 hrs at 4 °C, followed by addition of sepharose beads-coupled to protein A (Amersham Bioscience) for 1 hr at 4 °C. Immunoprecipitated Trk were washed in lysis buffer, denatured in sample loading buffer (1.67% SDS, 8.3% glycerol, 1.3% DTT (Sigma) trace bromophenol blue (Fisher)), boiled, run on a 7% SDS-PAGE, and transferred on a nitrocellulose membrane (Protran® Mandel, Keene, NH). Western blotting was performed using the pan-Trk 203 antibody.

Western Blotting: To characterize the TrkA signal transduction pathway, NGP cells were infected and treated as described above for 24 to 36 hrs in the presence of 50 ng/ml of NGF, then lysed, with NP-40 lysis buffer or RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 500 μM sodium vanadate and protease inhibitor cocktail). Cell lysates were equalized, denatured in sample loading buffer, boiled, run on 7% to 15% gradient SDS-PAGE (unless otherwise indicated), and transferred to nitrocellulose membrane. Membranes were blocked in 3% BSA (Calbiochem)/TBS-T (TBS containing 0,2% Teen-20 (Fisher)) for anti-phospho antibodies, or with milk blocking buffer (5% skim milk (Carnation), 1% BSA in TBS-T)

for 1 hr at room temperature (RT). Primary anti-phospho-antibodies were diluted in TBS-T and the other antibodies were diluted in blocking buffer, and incubated with the membrane overnight at 4 °C. Following washes, the membranes were incubated with secondary antibodies diluted in TBS-T for 1 hr at RT. Antibodies were visualized using the chemoluminescence ECL™ kit (Amersham Biosciences), according to the manufacturer guidelines. For reprobe analysis, the membranes were incubated with striping buffer (2% SDS; 62 mM Tris pH 6.8, 0.7% β-mercaptoethanol) for 15 minutes at 55 °C, rinsed several time in TBS, blocked and reprobed.

Densitometry analysis

The densitometry analysis was performed on scanned films with Total Lab 1.0 software (Amersham Biosciences). The intensity value for p53 bands were normalized by dividing their value by the intensity value for ERK of the same sample. To determine the change in p53 levels (in fold of uninfected cells) among the samples the normalized p53 values for each condition were divided by the normalized value of p53 of uninfected cells.

Immunostaining assays

NGP cells were counted, plated on poly-D-lysine coated 2 wells chamber slides (Nalgene, Naperville, IL), and infected as described above in presence of 50 ng/ml NGF. At 27 hours post-infection, media was removed, cells were washed with PBS (phosphate-buffered saline, BioWhittaker), fixed in 4% paraformaldehyde for 20 min, blocked in 20% normal goat serum, 1% bovine serum albumin (both from Jackson ImmunoResearch Laboratories) in PBS for 1 hr at room temperature. Primary antibody diluted in blocking solution was added overnight at 4° C. Cell were then incubated with secondary antibodies coupled to fluorochrome (diluted in blocking solution) for one hour at room temperature. Nuclei were stained with Hoechst and pictures were taken using an upright Zeiss microscope with a 40X lens.

Survival assays

MTT: Cells were plated in 48 well plate coated with rat tail collagen, infected as described, and NGF was added at time of infection. At 48 hrs post-infection, cell survival was assessed by incubating the cells for 1 hr with MTT reagent (Sigma) at 37 ˚C. Media was removed, cells were solubilized in isopropanol, and optical density, which corresponds to mitochondrial activity, was determined with an ELISA reader (OD 570 nm). *Trypan Blue exclusion assay*: NGP cells were plated on poly-D-lysine coated chamber slide and treated, as described, in the presence of neurotrophin. Cell survival was determined at 48 hrs post-treatment by incubating the cells with 20% trypan Blue (Sigma) added to the media for 10 min at 37 ˚C. Pictures of the cells were taken using an upright Zeiss microscope. The number of cells stained with trypan blue as well as the total number of cells per microscope field was calculated for 4 microscope fields. An average of 1500 cells was counted per condition. The percentage of cell death was calculated by dividing the number of dead cells by the total number of cells for each condition.

Statistical analysis

All statistical analyses were performed using Student t-Test or ANOVA, as indicated.

III. Discussion

Here we show that overexpression of the kinase-active TrkA neurotrophin receptor but not the kinase-defective TrkA (KDTrkA), induces the death of two different neuroblastoma cell lines. The overexpression of TrkA resulted in alterations in protein levels or activites of a number of proteins implicated in the regulation of apoptosis. These included increases in the levels of p53, decreases in the levels of Bcl-2, and in the activation of caspase-3. Furthermore, treatment with a broad caspase inhibitor or overexpression of the anti-apoptotic protein Bcl-X_L prevented TrkA from inducing cell death. These results strongly suggest that TrkA induces apoptotic cell death by a mechanism involving the modulation of pro- and anti-apoptotic proteins. While our results are consistent with TrkA causing apoptosis, we will need to perform cell death assay specific for apoptosis, such as the TUNEL assay, which specifically stains fragmented DNA, a characteristic of apoptotic cells.

The results confirmed the preliminary data of Dr. Lynne LeSauteur, suggesting that overexpression of TrkA using a TrkA-encoding recombinant adenovirus that was contaminated with wild-type (WT) adenovirus induced the death of neuroblastoma cells. Since the infection of NGP cells with WT Ad5 alone did not induce cell death, and the uncontaminated TrkA-encoding adenovirus efficiently induced cell death, we conclude that overexpression of TrkA is sufficient to induce cell death in NGP and 15N neuroblastoma cells. However, infection of NGP and 15N cells with equivalent MOIs of TrkA Ad5 resulted in different levels of cell death. This difference may be explained by a difference in TrkA expression levels in each cell type. In support of this explanation, we found that (1) using equivalent MOIs, the expression of TrkA was higher in NGP than in 15N cells and (2) TrkA expression using increasing MOIs of TrkA Ad5 induced the dose-dependent death of both NGP and 15N cells. However, from these experiments, we cannot exclude the possibility that 15N cells are more resistant to TrkA-induced cell death than NGP cells. This result, however, contrast with previous studies (Chen et al., 1990; Lavenius et al., 1995; Lucarelli et al., 1997; Matsushima and Bogenmann, 1993; Poluha et al., 1995) showing that overexpression of TrkA in neuroblastoma cell lines induces cell differentiation but not cell death. This discrepancy could be explained either

by the methods used (transient infection versus stable transfection) to assess the biological function of TrkA, or by the levels of TrkA overexpression achieved using adenovirus. In these previous studies, TrkA cDNA was stably transfected into NB cells, and the transfected cells were then selected and amplified prior to studying the biological action of TrkA. This method, which requires several weeks to obtain TrkA-expressing cell clones, is likely to select for NB cells that are resistant to TrkA-induced death. Therefore the cell death-inducing effect of TrkA would not be observed using this method. In contrast, overexpression of TrkA using a recombinant adenovirus does not select for neuroblastoma cells that could be resistant to TrkA-induced cell death, since most cells in a tissue culture dish are transiently infected with the recombinant virus. This method allows for high levels of TrkA expression. These levels are not likely to be toxic *per se*; similar levels of kinase-inactive TrkA or wild-type TrkB expression did not induce cell death.

MEK/ERK1/2 pathway is not required for TrkA-induced cell death.

TrkA has been shown to activate three major signal transduction cascades upon binding to NGF, the PLC-γ, the Ras/Raf/MEK/ERK1/2 and the PI-3K/Akt signaling pathways (Kaplan and Miller, 1997). Therefore we tested the possibility that one of these signaling cascades is implicated in mediating TrkA-induced cell death. Among these three signal transduction pathways, the preliminary data generated by Dr. LeSauteur with the contaminated TrkA Ad5, suggested that TrkA induced cell death via the MEK/ERK1/2 signaling cascade. However, our data shows that inhibition of the MEK/ERK signaling pathway using the pharmacological inhibitor U0126 did not prevent TrkA (expressed from uncontaminated TrkA Ad5) from inducing cell death. This contrasting result could be explained by the following observations. First, infection with the TrkA Ad5 contaminated with WT Ad5 is likely to lead to the expression of TrkA and the WT Ad5 E1 gene products, the E1A and E1B proteins. The E1B55K protein can interact with and inhibit p53 functions (Aloyz et al., 1998). Therefore, it is possible that co-expression of the WT Ad5 genes resulted in the partial inhibition of p53. Secondly, we generated data, not included in this thesis, showing that inhibition of MEK using the U0126 compound, which did not prevent TrkA from inducing cell death, prevented TrkA from inducing the

up-regulation of p53. This observation is consistent with previous reports showing that in neuroblastoma cells inhibition of the MEK/ERK1/2 pathway prevents the up-regulation of p53 caused by cell death inducers such as cisplatin (Park et al., 2001; Woessmann et al., 2002). Therefore, it is possible that, in the presence of E1A/E1B genes, the endogenously expressed p53 becomes neutralized, forcing TrkA to use MEK/ERK to up-regulate p53 levels to induce apoptosis. In contrast, in the absence of E1A/E1B proteins, the up-regulation of p53 could be dispensable for TrkA-induced cell death, as the endogenous levels of p53 might be sufficient to induce apoptosis. In this case, we hypothesize that TrkA causes the activation of p53 by inducing cellular re-localization to the nucleus. In support of this hypothesis, p53 has frequently been observed sequestered in the cytoplasm of neuroblastoma cells and cell lines (Moll et al., 1995; Moll et al., 1996). As a consequence, overexpression of TrkA might induce the re-localization of p53 from the cytoplasmic to nuclear compartments, thereby activating p53 downstream effectors. To test this hypothesis, it will be necessary to determine, by immunostaining, the endogenous localization of p53 in NGP cells and its localization following overexpression of TrkA. The above hypothesis is based upon the assumption that TrkA induces cell death via a p53 dependent mechanism. In future studies, we will need to determine whether TrkA will induce death in the absence of p53 for example, following treatment of the neuroblastoma cells with small interfering (siRNA), anti-sense RNA directed towards p53, or in the presence of p53 negative regulators such as the E1B55K protein (Aloyz et al., 1998), which inhibits p53 function. In support of this hypothesis, our preliminary results indicate that TrkA will not induce the death of a neuroblastoma cell line expressing a mutated non-functional p53 (data not shown).

JNK and p38MAPK are not activated following TrkA expression.

We next examined whether other signaling pathways implicated in apoptotic processes in neural tumor cells such as PC12 and medulloblastoma cells were activated by TrkA overexpression, including JNK and p38MAPK. Neither JNK nor p38MAPK was activated by TrkA expression in NB cells. This result contrasts with a recent study in PC12 cells, showing that overexpressed TrkA induced the activation of the MEK3/6-p38MAPK pathway at the same time points used in our study, and that activation of this

pathway was required for TrkA-induced cell death (Yan et al., 2002). Therefore, our results suggest that TrkA induces cell death of human neuroblastoma cells by a mechanism distinct from PC12 cells. This result also contrasts with the important role of JNK in the induction of apoptosis of sympathetic neurons (Aloyz et al., 1998; Miller et al., 2000). However, in this paradigm, JNK becomes activated following withdrawal of NGF and not following activation of TrkA. Therefore, our results suggest that the activation of JNK and p38MAPK proteins is not a mechanism used by TrkA to induce the death of human NB cells.

TrkA activates pRB activation and increases the expression of p53 and p21^{WAF}

We showed that in NGP cells, overexpression of TrkA resulted in the activation of pRB and in the up-regulation of p21^{WAF}, two important cell cycle repressors. Our time course experiments showed that pRB became hypo-phosphorylated at 24 hrs post-TrkA Ad5 infection, ie, 6 hrs prior to the peak in p21^{WAF} up-regulation. This suggests that the increase in hypo-phosphorylated levels of pRB might occur prior to the up-regulation of p21^{WAF}. This increase in activity and in up-regulation of these cell cycle repressors is consistent with previous studies showing that activation of over-expressed TrkA results in growth arrest in neuroblastoma cells (Lucarelli et al., 1997; Matsushima and Bogenmann, 1993; Poluha et al., 1995). However, it remains to be determined whether overexpression of TrkA induces cell cycle arrest in our neuroblastoma cells. This will be assessed by FACS analysis. In addition, several experiments will be required to determine the mechanism by which TrkA induces the up-regulation of hypo-phosphorylated pRB levels. One possible mechanism is that TrkA might suppress the activity of pRB negative regulators such as cyclin-dependent kinases (CDK) and N-Myc, which would prevent the inactivation of pRB. As a consequence, pRB will be active and will repress entry into the cell cycle. To test this hypothesis, it will be necessary to determine whether the increase in hypo-phosphorylated pRB is required by TrkA to induce cell cycle arrest. If activated pRB is required for TrkA-induced cell cycle arrest, then we will determine whether the catalytic activity of cdk as well as the protein levels and the localization of N-Myc protein are modulated following TrkA expression. If pRB activation is not required for TrkA-induced cell cycle arrest, we will investigate

whether TrkA modulates the activity of cdk or if it modulates the protein levels or localization (nuclear vs. cytoplasmic) of the N-Myc protein.

p53 is a potent cell cycle negative regulator as well as an important apoptosis inducer (Levine, 1997). Our results show that subsequent to TrkA expression, p53 levels increase in NGP cells. However, the mechanism by which TrkA causes this increase in p53 levels remains to be determined. One possibility is that TrkA induces stabilization of p53 protein by stimulating its phosphorylation. This could be tested by Western blotting using antibodies that recognize specific phosphorylated residues of p53 involved in stabilizing this protein, including anti-phosho p53 serine 46. Since p53 is a transcription factor, this increase in p53 is likely to result in the transcription of p53 gene targets such as the cyclin-cdk inhibitor, $p21^{WAF}$. Our observation that $p21^{WAF}$ up-regulation followed the up-regulation of p53 (figures 2.6C and 2.7E) is consistent with this model. However it will be necessary to perform experiments to confirm that p53 is the inducer of $p21^{WAF}$ in this model, since the p53 status, with regard to mutation and sub-cellular localization, is unknown in NGP. In these experiments, we would determine whether TrkA causes an increase in $p21^{WAF}$ levels when p53 function is inhibited by small interfering RNAs targeted to p53 mRNA or following the expression of the E1B55K protein. TrkA may also cause an accumulation or increase in the levels of p53 in the nucleus of NGP cells. Indeed, our preliminary data indicates that TrkA expression increases the levels of p53 in the cytoplasm and nucleus (data not shown).

Our pRB, p53 and $p21^{WAF}$ time course experiments suggest that pRB lies upstream of p53 and $p21^{WAF}$, as pRB became hypo-phosphorylated prior to the peak of p53 and $p21^{WAF}$ up-regulation. Consistent with this observation, pRB has been reported to be implicated in the death of sympathetic neurons deprived of NGF (Park et al., 1996; Park et al., 1997). However, in this signaling cascade, consisting of CDK4/6-pRB-p19ARF-p53, pRB becomes hyper-phosphorylated by CDK4/6, which contrast with the increase in hypo-phosphorylated pRB levels observed following TrkA over-expression in NGP cells. Therefore, if pRB is implicated in inducing TrkA-mediated cell death, it would do so in a manner distinct from sympathetic neurons. One mechanism by which pRB might induce the apoptosis of neuroblastoma cells is by dysregulating of the cell cycle. The activation of pRB might partially inhibit the cell cycle, which is constitutively

"on" due to the high levels of N-myc expression in many NB cell lines such as NGP (Nakagawara et al., 1994; Schwab et al., 1983), producing conflicting signals that might "confuse" the cells, and subsequently cause the induction of p53 and apoptosis. In future studies, we will determine the role of pRB in TrkA induced cell death by the following experiments. First, to determine whether activation of pRB is sufficient to induce cell death in neuroblastoma cells, the survival of NGP cells could be assessed following the over-expression of a dominant active pRB. If the expression of this pRB mutant induces cell death, we will determine whether this active mutant of pRB regulates the same death pathway as TrkA, consisting of the up-regulation of p53, down-regulation of Bcl-2 and caspase-3 cleavage. Secondly, to determine whether the activation of pRB is required by TrkA to induce cell death, the survival of NGP overexpressing TrkA and a dominantly inactive form of pRB could be assessed. If pRB is required for TrkA to induce cell death, the over-expression of a dominantly inactive pRB should prevent TrkA from causing cell death.

TrkA-decreases Bcl-2 protein levels

Our results showed that overexpression of TrkA resulted in a decrease in the levels of the Bcl-2 protein, a key anti-apoptotic protein for many cell types. Several potential mechanisms might account for this observation. First, we showed that TrkA induces the up-regulation of p53, and p53 has been reported to repress Bcl-2 transcription at the transcriptional level (Budhram-Mahadeo et al., 1999; Wu et al., 2001). Therefore it could be hypothesized that as a consequence of the up-regulation or activation of p53 by TrkA, the *de novo* expression of the Bcl-2 gene becomes repressed. This decrease in Bcl-2 transcription might explain the decrease in Bcl-2 protein levels observed following TrkA expression, which might be sufficient to induce apoptosis. This hypothesis could be tested by quantitative RT-PCR to determine the levels of Bcl-2 mRNA following TrkA expression. To determine whether the decrease in Bcl-2 levels is sufficient to induce apoptosis in these cells, survival of NGP cells could be assessed following the inhibition of Bcl-2 expression using anti-sense RNA or siRNA techniques.

A second mechanism by which TrkA could decrease the levels of Bcl-2 is by activation of caspases. Bcl-2 is a target of caspases (Thornberry and Lazebnik, 1998),

and cleavage of Bcl-2 by caspase-3 leads to an increase in caspase activation (Cheng et al., 1997). At the time point at which we evaluated the levels of Bcl-2, we showed that caspase-3 was activated. Therefore, it is possible that the observed decrease in Bcl-2 was caused by its cleavage by caspases. To determine whether the decrease in Bcl-2 levels is caused by caspase activation, we will need to assess the Bcl-2 protein levels in NGP cells overexpressing TrkA before and after treatment with a broad-spectrum caspase inhibitor. If the caspase inhibitor does not prevent the down-regulation of Bcl-2, that will imply that the activation of caspases is not implicated in the modulation of Bcl-2. In contrast, if the treatment with the caspase inhibitor prevents the decrease in Bcl-2 levels, then this suggests that the Bcl-2 protein is regulated by caspase activity. In addition, we could determine the levels of Bcl-2 at time points prior to or after caspase activation. This experiment will allow us to determine where Bcl-2 acts in the p53/caspase-3 pathway.

Our results showing TrkA-induced down-regulation of Bcl-2 contrasts with a previous report showing that activation of TrkA in PC12 results in the up-regulation of Bcl-2 (Liu et al., 1999). These different results might reflect fundamental differences in the signaling cascades activated by TrkA in NB vs. PC12 cells.

IV. Conclusion:

The data presented here show that overexpression of the TrkA/Nerve Growth Factor receptor using a recombinant adenovirus induces the death of two neuroblastoma cell lines. This result confirms the preliminary data generated by Dr. Lynne LeSauteur in the laboratory of Dr. David Kaplan that overexpression of TrkA (using a WT adenovirus contaminated TrkA Ad5) induced the death of established and primary neuroblastoma cell lines, and shows that expression of WT Ad5 E1A/E1B genes is dispensible for TrkA to induce the death of these cells. Our results also suggest a mechanism for TrkA-mediated neuroblastoma cell death. TrkA expression modulated the levels or activities of key regulators of the apoptotic pathway: p53 was up-regulated, Bcl-2 was down-regulated, and caspase-3 was cleaved and activated. In addition, we showed that overexpression of the anti-apopotic protein Bcl-X_L or the presence of the broad caspase

inhibitor, z-VAD-fmk prevented caspase-3 cleavage and protected the cells against TrkA-induced death. Even though cell death assays such as TUNEL will need to be performed in order to confirm that TrkA induces cell death by apoptosis, these results strongly suggest that TrkA induces apoptotic death in our neuroblastoma cell lines.

The up-regulation of p53 by TrkA suggests that p53 plays a role in TrkA-induced cell death. However, it remains to be determined whether p53 is essential for TrkA to induce death. This could be tested in NGP cells in which p53 expression is suppressed by short interfering RNA (siRNA). If p53 expression is not required for TrkA-induced death, it would be logical to ask whether other p53-family members, such as p63 or p73, could be responsible for mediating TrkA-induced death.

To determine the mechanism by which TrkA leads to the up-regulation of p53, down-regulation of Bcl-2 and activation of caspase-3, we looked at the phosphorylation status of three known MAP kinases, ERK1/2, JNK, and p38 MAPK, which have been reported to upregulate (ERK) or phosphorylate (JNK and p38 MAPK) p53, as an indicator of their activity. We showed that TrkA activation leads to the robust phosphorylation of ERK1/2, but treatment with MEK inhibitor showed that activation of ERK is dispensable for TrkA-induced cell death. Therefore these results suggest that the phosphorylation of JNK, p38 MAPK and ERK are not required for TrkA-induced death of our neuroblastoma cell lines.

As a consequence, further studies will be required to determine the mechanism or the signaling cascade activated by TrkA, and that is responsible for the modulation of p53, Bcl-2 and caspase-3. Such a pathway may involve a Ras-dependent, but ERK1/2 independent pathway, such as the one described in TrkA-induced cell death of medulloblastoma (Chou et al., 2000). In addition, p53 is frequently sequestered in the cytoplasm of neuroblastoma cells, and TrkA has been shown to directly interact with p53 in PC12 cells. Therefore, TrkA may induce cell death by interacting with p53 to induce p53 activation, or by causing p53 to translocate to the nucleus. Another mechanism by which TrkA may induce cell death is by causing a cell cycle conflict. Our results show that high expression of TrkA induces an increase in the levels of hypo-phosphorylated (active) pRB and the protein levels of $p21^{WAF}$, two cell cycle inhibitors.

The expression of a strong cell cycle inhibitor such as TrkA in cycling tumor cells may contribute to or enhance the pro-apoptotic action of this protein.

Finally, these results identify apoptosis as a novel biological response of TrkA. This is consistent with TrkA as a good prognosis marker for neuroblastoma, and suggests that it might do so by inducing the death of the tumor cells. In addition, our results suggest that therapies based upon TrkA overexpression or stimulation will be efficacious for the treatment of neuroblastoma. However, future studies will be required to determine whether this TrkA-based therapy will be efficacious in neuroblastomas expressing a mutated or non-functional p53. In addition, it will be necessary to determine whether neuroblastoma tumors with defects in their apoptotic pathways, such as those tumors overexpressing Bcl-X_L or survivin, would be resistant to TrkA-induced cell death. These studies along with the results reported here will determine whether a TrkA-based therapy will be an effective therapy for all or a subset of neuroblastoma tumors.

V. References:

Aloyz, R.S., Bamji, S.X., Pozniak, C.D., Toma, J.G., Atwal, J., Kaplan, D.R. and Miller, F.D. (1998) p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. *J Cell Biol*, **143**, 1691-1703.

Anderson, C.N. and Tolkovsky, A.M. (1999) A role for MAPK/ERK in sympathetic neuron survival: protection against a p53-dependent, JNK-independent induction of apoptosis by cytosine arabinoside. *J Neurosci*, **19**, 664-673.

Aoyama, M., Asai, K., Shishikura, T., Kawamoto, T., Miyachi, T., Yokoi, T., Togari, H., Wada, Y., Kato, T. and Nakagawara, A. (2001) Human neuroblastomas with unfavorable biologies express high levels of brain-derived neurotrophic factor mRNA and a variety of its variants. *Cancer Lett*, **164**, 51-60.

Atwal, J.K., Massie, B., Miller, F.D. and Kaplan, D.R. (2000) The TrkB-Shc site signals neuronal survival and local axon growth via MEK and P13-kinase. *Neuron*, **27**, 265-277.

Bamji, S.X., Majdan, M., Pozniak, C.D., Belliveau, D.J., Aloyz, R., Kohn, J., Causing, C.G. and Miller, F.D. (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. *J Cell Biol*, **140**, 911-923.

Banelli, B., Casciano, I., Croce, M., Di Vinci, A., Gelvi, I., Pagnan, G., Brignole, C., Allemanni, G., Ferrini, S., Ponzoni, M. and Romani, M. (2002) Expression and methylation of CASP8 in neuroblastoma: identification of a promoter region. *Nat Med*, **8**, 1333-1335; author reply 1335.

Barbacid, M. (1995) Neurotrophic factors and their receptors. *Curr Opin Cell Biol*, **7**, 148-155.

Birren, S.J., Lo, L. and Anderson, D.J. (1993) Sympathetic neuroblasts undergo a developmental switch in trophic dependence. *Development*, **119**, 597-610.

Bogenmann, E., Torres, M. and Matsushima, H. (1995) Constitutive N-myc gene expression inhibits trkA mediated neuronal differentiation. *Oncogene*, **10**, 1915-1925.

Bohn, M.C., Goldstein, M. and Black, I.B. (1981) Role of glucocorticoids in expression of the adrenergic phenotype in rat embryonic adrenal gland. *Dev Biol*, **82**, 1-10.

Bown, N. (2001) Neuroblastoma tumour genetics: clinical and biological aspects. *J Clin Pathol*, **54**, 897-910.

Bown, N., Cotterill, S., Lastowska, M., O'Neill, S., Pearson, A.D., Plantaz, D., Meddeb, M., Danglot, G., Brinkschmidt, C., Christiansen, H., Laureys, G., Speleman, F., Nicholson, J., Bernheim, A., Betts, D.R., Vandesompele, J. and Van Roy, N. (1999) Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. *N Engl J Med*, **340**, 1954-1961.

Brodeur, G.M. (2003) Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer*, **3**, 203-216.

Brodeur, G.M., Green, A.A., Hayes, F.A., Williams, K.J., Williams, D.L. and Tsiatis, A.A. (1981) Cytogenetic features of human neuroblastomas and cell lines. *Cancer Research*, **41**, 4678-4686.

Brodeur, G.M., Nakagawara, A., Yamashiro, D.J., Ikegaki, N., Liu, X.G., Azar, C.G., Lee, C.P. and Evans, A.E. (1997) Expression of TrkA, TrkB and TrkC in human neuroblastomas. *J Neurooncol*, **31**, 49-55.

Brodeur, G.M., Seeger, R.C., Schwab, M., Varmus, H.E. and Bishop, J.M. (1984) Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science*, **224**, 1121-1124.

Brodeur, G.M., Sekhon, G. and Goldstein, M.N. (1977) Chromosomal aberrations in human neuroblastomas. *Cancer*, **40**, 2256-2263.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J. and Greenberg, M.E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*, **96**, 857-868.

Budhram-Mahadeo, V., Morris, P.J., Smith, M.D., Midgley, C.A., Boxer, L.M. and Latchman, D.S. (1999) p53 suppresses the activation of the Bcl-2 promoter by the Brn-3a POU family transcription factor. *J Biol Chem*, **274**, 15237-15244.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X. and Wang, X. (1999) Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol*, **15**, 269-290.

Bunone, G., Mariotti, A., Compagni, A., Morandi, E. and Della Valle, G. (1997) Induction of apoptosis by p75 neurotrophin receptor in human neuroblastoma cells. *Oncogene*, **14**, 1463-1470.

Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S. and Reed, J.C. (1998) Regulation of cell death protease caspase-9 by phosphorylation. *Science*, **282**, 1318-1321.

Caron, H. (1995) Allelic loss of chromosome 1 and additional chromosome 17 material are both unfavourable prognostic markers in neuroblastoma. *Med Pediatr Oncol*, **24**, 215-221.

Caron, H., van Sluis, P., de Kraker, J., Bokkerink, J., Egeler, M., Laureys, G., Slater, R., Westerveld, A., Voute, P.A. and Versteeg, R. (1996) Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. *N Engl J Med*, **334**, 225-230.

Chen, J., Chattopadhyay, B., Venkatakrishnan, G. and Ross, A.H. (1990) Nerve growth factor-induced differentiation of human neuroblastoma and neuroepithelioma cell lines. *Cell Growth Differ*, **1**, 79-85.

Cheng, E.H., Kirsch, D.G., Clem, R.J., Ravi, R., Kastan, M.B., Bedi, A., Ueno, K. and Hardwick, J.M. (1997) Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science*, **278**, 1966-1968.

Chou, T.T., Trojanowski, J.Q. and Lee, V.M. (2000) A novel apoptotic pathway induced by nerve growth factor-mediated TrkA activation in medulloblastoma. *J Biol Chem*, **275**, 565-570.

Ciccarone, V., Spengler, B.A., Meyers, M.B., Biedler, J.L. and Ross, R.A. (1989) Phenotypic diversification in human neuroblastoma cells: expression of distinct neural crest lineages. *Cancer Res*, **49**, 219-225.

Cortazzo, M.H., Kassis, E.S., Sproul, K.A. and Schor, N.F. (1996) Nerve growth factor (NGF)-mediated protection of neural crest cells from antimitotic agent-induced apoptosis: the role of the low-affinity NGF receptor. *J Neurosci*, **16**, 3895-3899.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M. and Hemmings, B.A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*, **378**, 785-789.

Cui, H., Schroering, A. and Ding, H.F. (2002) p53 mediates DNA damaging drug-induced apoptosis through a caspase-9-dependent pathway in SH-SY5Y neuroblastoma cells. *Mol Cancer Ther*, **1**, 679-686.

Cunningham, M.E., Stephens, R.M., Kaplan, D.R. and Greene, L.A. (1997) Autophosphorylation of activation loop tyrosines regulates signaling by the TRK nerve growth factor receptor. *J Biol Chem*, **272**, 10957-10967.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. and Greenberg, M.E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*, **91**, 231-241.

Dole, M.G., Jasty, R., Cooper, M.J., Thompson, C.B., Nunez, G. and Castle, V.P. (1995) Bcl-xL is expressed in neuroblastoma cells and modulates chemotherapy-induced apoptosis. *Cancer Res*, **55**, 2576-2582.

Edsjo, A., Hallberg, B., Fagerstrom, S., Larsson, C., Axelson, H. and Pahlman, S. (2001) Differences in early and late responses between neurotrophin-stimulated trkA- and trkC-transfected SH-SY5Y neuroblastoma cells. *Cell Growth Differ*, **12**, 39-50.

Eggert, A., Grotzer, M.A., Zuzak, T.J., Wiewrodt, B.R., Ho, R., Ikegaki, N. and Brodeur, G.M. (2001) Resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. *Cancer Res*, **61**, 1314-1319.

Eggert, A., Ho, R., Ikegaki, N., Liu, X.G. and Brodeur, G.M. (2000a) Different effects of TrkA expression in neuroblastoma cell lines with or without MYCN amplification. *Med Pediatr Oncol*, **35**, 623-627.

Eggert, A., Ikegaki, N., Liu, X., Chou, T.T., Lee, V.M., Trojanowski, J.Q. and Brodeur, G.M. (2000b) Molecular dissection of TrkA signal transduction pathways mediating differentiation in human neuroblastoma cells. *Oncogene*, **19**, 2043-2051.

Eggert, A., Sieverts, H., Ikegaki, N. and Brodeur, G.M. (2000c) p75 mediated apoptosis in neuroblastoma cells is inhibited by expression of TrkA. *Med Pediatr Oncol*, **35**, 573-576.

ElShamy, W.M., Fridvall, L.K. and Ernfors, P. (1998) Growth arrest failure, G1 restriction point override, and S phase death of sensory precursor cells in the absence of neurotrophin-3. *Neuron*, **21**, 1003-1015.

Encinas, M., Iglesias, M., Llecha, N. and Comella, J.X. (1999) Extracellular-regulated kinases and phosphatidylinositol 3-kinase are involved in brain-derived neurotrophic factor-mediated survival and neuritogenesis of the neuroblastoma cell line SH-SY5Y. *J Neurochem*, **73**, 1409-1421.

Fagan, A.M., Zhang, H., Landis, S., Smeyne, R.J., Silos-Santiago, I. and Barbacid, M. (1996) TrkA, but not TrkC, receptors are essential for survival of sympathetic neurons in vivo. *J Neurosci*, **16**, 6208-6218.

Farinas, I. (1999) Neurotrophin actions during the development of the peripheral nervous system. *Microsc Res Tech*, **45**, 233-242.

Fesik, S.W. and Shi, Y. (2001) Structural biology. Controlling the caspases. *Science*, **294**, 1477-1478.

Fong, C.T., Dracopoli, N.C., White, P.S., Merrill, P.T., Griffith, R.C., Housman, D.E. and Brodeur, G.M. (1989) Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification. *Proc Natl Acad Sci U S A*, **86**, 3753-3757.

Fulda, S., Sieverts, H., Friesen, C., Herr, I. and Debatin, K.M. (1997) The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. *Cancer Res*, **57**, 3823-3829.

Gehring, M., Berthold, F., Edler, L., Schwab, M. and Amler, L.C. (1995) The 1p deletion is not a reliable marker for the prognosis of patients with neuroblastoma. *Cancer Res*, **55**, 5366-5369.

Gestblom, C., Hoehner, J.C., Hedborg, F., Sandstedt, B. and Pahlman, S. (1997) In vivo spontaneous neuronal to neuroendocrine lineage conversion in a subset of neuroblastomas. *Am J Pathol*, **150**, 107-117.

Hanahan, D. and Weinberg, R.A. (2000) The hallmarks of cancer. *Cell*, **100**, 57-70.

Hempstead, B.L. (2002) The many faces of p75NTR. *Curr Opin Neurobiol*, **12**, 260-267.

Hempstead, B.L., Rabin, S.J., Kaplan, L., Reid, S., Parada, L.F. and Kaplan, D.R. (1992) Overexpression of the trk tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. *Neuron*, **9**, 883-896.

Henderson, C.E. (1996) Role of neurotrophic factors in neuronal development. *Curr Opin Neurobiol*, **6**, 64-70.

Hino, T., Sugimoto, T., Matsumura, T., Horii, Y., Inazawa, J. and Sawada, T. (1989) Diverse responses to retinoid in morphological differentiation, tumorigenesis and N-myc expression in human neuroblastoma sublines. *Int J Cancer*, **44**, 286-291.

Hiyama, E., Hiyama, K., Yokoyama, T., Matsuura, Y., Piatyszek, M.A. and Shay, J.W. (1995) Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat Med*, **1**, 249-255.

Ho, R., Eggert, A., Hishiki, T., Minturn, J.E., Ikegaki, N., Foster, P., Camoratto, A.M., Evans, A.E. and Brodeur, G.M. (2002) Resistance to chemotherapy mediated by TrkB in neuroblastomas. *Cancer Res*, **62**, 6462-6466.

Hoehner, J.C., Gestblom, C., Hedborg, F., Sandstedt, B., Olsen, L. and Pahlman, S. (1996) A developmental model of neuroblastoma: differentiating stroma-poor tumors' progress along an extra-adrenal chromaffin lineage. *Lab Invest*, **75**, 659-675.

Hoehner, J.C., Olsen, L., Sandstedt, B., Kaplan, D.R. and Pahlman, S. (1995) Association of neurotrophin receptor expression and differentiation in human neuroblastoma. *Am J Pathol*, **147**, 102-113.

Holgado-Madruga, M., Moscatello, D.K., Emlet, D.R., Dieterich, R. and Wong, A.J. (1997) Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. *Proc Natl Acad Sci U S A*, **94**, 12419-12424.

Imamura, J., Bartram, C.R., Berthold, F., Harms, D., Nakamura, H. and Koeffler, H.P. (1993) Mutation of the p53 gene in neuroblastoma and its relationship with N-myc amplification. *Cancer Res*, **53**, 4053-4058.

Islam, A., Kageyama, H., Takada, N., Kawamoto, T., Takayasu, H., Isogai, E., Ohira, M., Hashizume, K., Kobayashi, H., Kaneko, Y. and Nakagawara, A. (2000) High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. *Oncogene*, **19**, 617-623.

Jaboin, J., Kim, C.J., Kaplan, D.R. and Thiele, C.J. (2002) Brain-derived neurotrophic factor activation of TrkB protects neuroblastoma cells from chemotherapy-induced apoptosis via phosphatidylinositol 3'-kinase pathway. *Cancer Res*, **62**, 6756-6763.

Kanamoto, T., Mota, M., Takeda, K., Rubin, L.L., Miyazono, K., Ichijo, H. and Bazenet, C.E. (2000) Role of apoptosis signal-regulating kinase in regulation of the c-Jun N-terminal kinase pathway and apoptosis in sympathetic neurons. *Mol Cell Biol*, **20**, 196-204.

Kaneko, Y., Kanda, N., Maseki, N., Nakachi, K., Takeda, T., Okabe, I. and Sakurai, M. (1990) Current urinary mass screening for catecholamine metabolites at 6 months of age may be detecting only a small portion of high-risk neuroblastomas: a chromosome and N-myc amplification study. *J Clin Oncol*, **8**, 2005-2013.

Kaneko, Y., Kanda, N., Maseki, N., Sakurai, M., Tsuchida, Y., Takeda, T. and Okabe, I. (1987) Different karyotypic patterns in early and advanced stage neuroblastomas. *Cancer Res*, **47**, 311-318.

Kaplan, D.R., Matsumoto, K., Lucarelli, E. and Thiele, C.J. (1993) Induction of TrkB by retinoic acid mediates biologic responsiveness to BDNF and differentiation of human neuroblastoma cells. Eukaryotic Signal Transduction Group. *Neuron*, **11**, 321-331.

Kaplan, D.R. and Miller, F.D. (1997) Signal transduction by the neurotrophin receptors. *Curr Opin Cell Biol*, **9**, 213-221.

Kaplan, D.R. and Miller, F.D. (2000) Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol*, **10**, 381-391.

Keshelava, N., Zuo, J.J., Chen, P., Waidyaratne, S.N., Luna, M.C., Gomer, C.J., Triche, T.J. and Reynolds, C.P. (2001) Loss of p53 function confers high-level multidrug resistance in neuroblastoma cell lines. *Cancer Res*, **61**, 6185-6193.

Kim, J.Y., Sutton, M.E., Lu, D.J., Cho, T.A., Goumnerova, L.C., Goritchenko, L., Kaufman, J.R., Lam, K.K., Billet, A.L., Tarbell, N.J., Wu, J., Allen, J.C., Stiles, C.D., Segal, R.A. and Pomeroy, S.L. (1999) Activation of neurotrophin-3 receptor TrkC induces apoptosis in medulloblastomas. *Cancer Res*, **59**, 711-719.

Knoepfler, P.S., Cheng, P.F. and Eisenman, R.N. (2002) N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. *Genes Dev*, **16**, 2699-2712.

Kohn, J., Aloyz, R.S., Toma, J.G., Haak-Frendscho, M. and Miller, F.D. (1999) Functionally antagonistic interactions between the TrkA and p75 neurotrophin receptors regulate sympathetic neuron growth and target innervation. *J Neurosci*, **19**, 5393-5408.

Komuro, H., Hayashi, Y., Kawamura, M., Hayashi, K., Kaneko, Y., Kamoshita, S., Hanada, R., Yamamoto, K., Hongo, T., Yamada, M. and et al. (1993) Mutations of the p53 gene are involved in Ewing's sarcomas but not in neuroblastomas. *Cancer Res*, **53**, 5284-5288.

Lachyankar, M.B., Condon, P.J., Daou, M.C., De, A.K., Levine, J.B., Obermeier, A. and Ross, A.H. (2003) Novel functional interactions between Trk kinase and p75 neurotrophin receptor in neuroblastoma cells. *J Neurosci Res*, **71**, 157-172.

Larcher, J.C., Basseville, M., Vayssiere, J.L., Cordeau-Lossouarn, L., Croizat, B. and Gros, F. (1992) Growth inhibition of N1E-115 mouse neuroblastoma cells by c-myc or N-myc antisense oligodeoxynucleotides causes limited differentiation but is not coupled to neurite formation. *Biochem Biophys Res Commun*, **185**, 915-924.

Lavenius, E., Gestblom, C., Johansson, I., Nanberg, E. and Pahlman, S. (1995) Transfection of TRK-A into human neuroblastoma cells restores their ability to differentiate in response to nerve growth factor. *Cell Growth Differ*, **6**, 727-736.

Lee, R., Kermani, P., Teng, K.K. and Hempstead, B.L. (2001) Regulation of cell survival by secreted proneurotrophins. *Science*, **294**, 1945-1948.

Levine, A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323-331.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, **91**, 479-489.

Liu, W.S. and Heckman, C.A. (1998) The sevenfold way of PKC regulation. *Cell Signal*, **10**, 529-542.

Liu, Y.Z., Boxer, L.M. and Latchman, D.S. (1999) Activation of the Bcl-2 promoter by nerve growth factor is mediated by the p42/p44 MAPK cascade. *Nucleic Acids Res*, **27**, 2086-2090.

Loeb, D.M., Stephens, R.M., Copeland, T., Kaplan, D.R. and Greene, L.A. (1994) A Trk nerve growth factor (NGF) receptor point mutation affecting interaction with phospholipase C-gamma 1 abolishes NGF-promoted peripherin induction but not neurite outgrowth. *J Biol Chem*, **269**, 8901-8910.

Look, A.T., Hayes, F.A., Shuster, J.J., Douglass, E.C., Castleberry, R.P., Bowman, L.C., Smith, E.I. and Brodeur, G.M. (1991) Clinical relevance of tumor cell ploidy and N-myc gene amplification in childhood neuroblastoma: a Pediatric Oncology Group study. *J Clin Oncol*, **9**, 581-591.

Lucarelli, E., Kaplan, D. and Thiele, C.J. (1997) Activation of trk-A but not trk-B signal transduction pathway inhibits growth of neuroblastoma cells. *Eur J Cancer*, **33**, 2068-2070.

Maris, J.M., Weiss, M.J., Guo, C., Gerbing, R.B., Stram, D.O., White, P.S., Hogarty, M.D., Sulman, E.P., Thompson, P.M., Lukens, J.N., Matthay, K.K., Seeger, R.C. and Brodeur, G.M. (2000) Loss of heterozygosity at 1p36 independently predicts for disease progression but not decreased overall survival probability in neuroblastoma patients: a Children's Cancer Group study. *J Clin Oncol*, **18**, 1888-1899.

Martinsson, T., Sjoberg, R.M., Hedborg, F. and Kogner, P. (1995) Deletion of chromosome 1p loci and microsatellite instability in neuroblastomas analyzed with short-tandem repeat polymorphisms. *Cancer Res*, **55**, 5681-5686.

Massie, B., Dionne, J., Lamarche, N., Fleurent, J. and Langelier, Y. (1995) Improved adenovirus vector provides herpes simplex virus ribonucleotide reductase R1 and R2 subunits very efficiently. *Biotechnology (N Y)*, **13**, 602-608.

Matsumoto, K., Wada, R.K., Yamashiro, J.M., Kaplan, D.R. and Thiele, C.J. (1995) Expression of brain-derived neurotrophic factor and p145TrkB affects survival, differentiation, and invasiveness of human neuroblastoma cells. *Cancer Res*, **55**, 1798-1806.

Matsushima, H. and Bogenmann, E. (1993) Expression of trkA cDNA in neuroblastomas mediates differentiation in vitro and in vivo. *Mol Cell Biol*, **13**, 7447-7456.

Mazzoni, I.E., Said, F.A., Aloyz, R., Miller, F.D. and Kaplan, D. (1999) Ras regulates sympathetic neuron survival by suppressing the p53-mediated cell death pathway. *J Neurosci*, **19**, 9716-9727.

Middlemas, D.S., Kihl, B.K., Zhou, J. and Zhu, X. (1999) Brain-derived neurotrophic factor promotes survival and chemoprotection of human neuroblastoma cells. *J Biol Chem*, **274**, 16451-16460.

Miller, F.D. and Kaplan, D.R. (2001) Neurotrophin signalling pathways regulating neuronal apoptosis. *Cell Mol Life Sci*, **58**, 1045-1053.

Miller, F.D., Pozniak, C.D. and Walsh, G.S. (2000) Neuronal life and death: an essential role for the p53 family. *Cell Death Differ*, **7**, 880-888.

Minichiello, L., Calella, A.M., Medina, D.L., Bonhoeffer, T., Klein, R. and Korte, M. (2002) Mechanism of TrkB-mediated hippocampal long-term potentiation. *Neuron*, **36**, 121-137.

Miyashita, T. and Reed, J.C. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell*, **80**, 293-299.

Moll, U.M., LaQuaglia, M., Benard, J. and Riou, G. (1995) Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. *Proc Natl Acad Sci U S A*, **92**, 4407-4411.

Moll, U.M., Ostermeyer, A.G., Haladay, R., Winkfield, B., Frazier, M. and Zambetti, G. (1996) Cytoplasmic sequestration of wild-type p53 protein impairs the G1 checkpoint after DNA damage. *Mol Cell Biol*, **16**, 1126-1137.

Moroni, M.C., Hickman, E.S., Denchi, E.L., Caprara, G., Colli, E., Cecconi, F., Muller, H. and Helin, K. (2001) Apaf-1 is a transcriptional target for E2F and p53. *Nat Cell Biol*, **3**, 552-558.

Nagata, S. (1997) Apoptosis by death factor. *Cell*, **88**, 355-365.

Nagata, S. (2000) Apoptotic DNA fragmentation. *Exp Cell Res*, **256**, 12-18.

Nakagawara, A., Arima, M., Azar, C.G., Scavarda, N.J. and Brodeur, G.M. (1992) Inverse relationship between trk expression and N-myc amplification in human neuroblastomas. *Cancer Res*, **52**, 1364-1368.

Nakagawara, A., Arima-Nakagawara, M., Scavarda, N.J., Azar, C.G., Cantor, A.B. and Brodeur, G.M. (1993) Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. *N Engl J Med*, **328**, 847-854.

Nakagawara, A., Azar, C.G., Scavarda, N.J. and Brodeur, G.M. (1994) Expression and function of TRK-B and BDNF in human neuroblastomas. *Mol Cell Biol*, **14**, 759-767.

Nakano, K. and Vousden, K.H. (2001) PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell*, **7**, 683-694.

Naumann, T., Casademunt, E., Hollerbach, E., Hofmann, J., Dechant, G., Frotscher, M. and Barde, Y.A. (2002) Complete deletion of the neurotrophin receptor p75NTR leads to long-lasting increases in the number of basal forebrain cholinergic neurons. *J Neurosci*, **22**, 2409-2418.

Nicholson, D.W. (2001) Apoptosis. Baiting death inhibitors. *Nature*, **410**, 33-34.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T. and Tanaka, N. (2000) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science*, **288**, 1053-1058.

Olsson, A.K. and Nanberg, E. (2001) A functional role for ERK in gene induction, but not in neurite outgrowth in differentiating neuroblastoma cells. *Exp Cell Res*, **265**, 21-30.

Olsson, A.K., Vadhammar, K. and Nanberg, E. (2000) Activation and protein kinase C-dependent nuclear accumulation of ERK in differentiating human neuroblastoma cells. *Exp Cell Res*, **256**, 454-467.

Pap, M. and Cooper, G.M. (1998) Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. *J Biol Chem*, **273**, 19929-19932.

Park, D.S., Farinelli, S.E. and Greene, L.A. (1996) Inhibitors of cyclin-dependent kinases promote survival of post-mitotic neuronally differentiated PC12 cells and sympathetic neurons. *J Biol Chem*, **271**, 8161-8169.

Park, D.S., Levine, B., Ferrari, G. and Greene, L.A. (1997) Cyclin dependent kinase inhibitors and dominant negative cyclin dependent kinase 4 and 6 promote survival of NGF-deprived sympathetic neurons. *J Neurosci*, **17**, 8975-8983.

Park, J.R. and Hockenbery, D.M. (1996) BCL-2, a novel regulator of apoptosis. *J Cell Biochem*, **60**, 12-17.

Park, S.A., Park, H.J., Lee, B.I., Ahn, Y.H., Kim, S.U. and Choi, K.S. (2001) Bcl-2 blocks cisplatin-induced apoptosis by suppression of ERK-mediated p53 accumulation in B104 cells. *Brain Res Mol Brain Res*, **93**, 18-26.

Poluha, W., Poluha, D.K. and Ross, A.H. (1995) TrkA neurogenic receptor regulates differentiation of neuroblastoma cells. *Oncogene*, **10**, 185-189.

Poulaki, V., Mitsiades, N., Romero, M.E. and Tsokos, M. (2001) Fas-mediated apoptosis in neuroblastoma requires mitochondrial activation and is inhibited by FLICE inhibitor protein and Bcl-2. *Cancer Res*, **61**, 4864-4872.

Pozniak, C.D., Radinovic, S., Yang, A., McKeon, F., Kaplan, D.R. and Miller, F.D. (2000) An anti-apoptotic role for the p53 family member, p73, during developmental neuron death. *Science*, **289**, 304-306.

Putcha, G.V., Moulder, K.L., Golden, J.P., Bouillet, P., Adams, J.A., Strasser, A. and Johnson, E.M. (2001) Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. *Neuron*, **29**, 615-628.

Qian, X., Riccio, A., Zhang, Y. and Ginty, D.D. (1998) Identification and characterization of novel substrates of Trk receptors in developing neurons. *Neuron*, **21**, 1017-1029.

Rebbaa, A., Chou, P.M., Emran, M. and Mirkin, B.L. (2001) Doxorubicin-induced apoptosis in caspase-8-deficient neuroblastoma cells is mediated through direct action on mitochondria. *Cancer Chemother Pharmacol*, **48**, 423-428.

Reynolds, C.P., Zuo, J.J., Kim, N.W., Wang, H., Lukens, J.N., Matthay, K.K. and Seeger, R.C. (1997) Telomerase expression in primary neuroblastomas. *Eur J Cancer*, **33**, 1929-1931.

Rui, L., Herrington, J. and Carter-Su, C. (1999) SH2-B is required for nerve growth factor-induced neuronal differentiation. *J Biol Chem*, **274**, 10590-10594.

Rustgi, A.K., Dyson, N. and Bernards, R. (1991) Amino-terminal domains of c-myc and N-myc proteins mediate binding to the retinoblastoma gene product. *Nature*, **352**, 541-544.

Sawai, S., Shimono, A., Wakamatsu, Y., Palmes, C., Hanaoka, K. and Kondoh, H. (1993) Defects of embryonic organogenesis resulting from targeted disruption of the N-myc gene in the mouse. *Development*, **117**, 1445-1455.

Scala, S., Wosikowski, K., Giannakakou, P., Valle, P., Biedler, J.L., Spengler, B.A., Lucarelli, E., Bates, S.E. and Thiele, C.J. (1996) Brain-derived neurotrophic factor protects neuroblastoma cells from vinblastine toxicity. *Cancer Res*, **56**, 3737-3742.

Schwab, M., Alitalo, K., Klempnauer, K.H., Varmus, H.E., Bishop, J.M., Gilbert, F., Brodeur, G., Goldstein, M. and Trent, J. (1983) Amplified DNA with limited

homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature*, **305**, 245-248.

Schwab, M., Shimada, H., Joshi, V. and Brodeur, G.M. (2000) Neuroblastic tumours of adrenal gland and sympathetic nervous system. In Kleihues, P.a.C., W.K. (ed.), *Tumours of the nervous system*. International Agency for Research on Cancer, Lyon, pp. 153-161.

Seeger, R.C., Brodeur, G.M., Sather, H., Dalton, A., Siegel, S.E., Wong, K.Y. and Hammond, D. (1985) Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. *N Engl J Med*, **313**, 1111-1116.

Serbedzija, G.N., Fraser, S.E. and Bronner-Fraser, M. (1990) Pathways of trunk neural crest cell migration in the mouse embryo as revealed by vital dye labelling. *Development*, **108**, 605-612.

Stanton, B.R. and Parada, L.F. (1992) The N-myc proto-oncogene: developmental expression and in vivo site-directed mutagenesis. *Brain Pathol*, **2**, 71-83.

Stanton, B.R., Perkins, A.S., Tessarollo, L., Sassoon, D.A. and Parada, L.F. (1992) Loss of N-myc function results in embryonic lethality and failure of the epithelial component of the embryo to develop. *Genes Dev*, **6**, 2235-2247.

Streutker, C.J., Thorner, P., Fabricius, N., Weitzman, S. and Zielenska, M. (2001) Telomerase activity as a prognostic factor in neuroblastomas. *Pediatr Dev Pathol*, **4**, 62-67.

Sun, L., Fuselier, J.A., Murphy, W.A. and Coy, D.H. (2002) Antisense peptide nucleic acids conjugated to somatostatin analogs and targeted at the n-myc oncogene display enhanced cytotoxity to human neuroblastoma IMR32 cells expressing somatostatin receptors. *Peptides*, **23**, 1557-1565.

Svensson, K. and Larsson, C. (2003) A protein kinase Cbeta inhibitor attenuates multidrug resistance of neuroblastoma cells. *BMC Cancer*, **3**, 10.

Svensson, T., Ryden, M., Schilling, F.H., Dominici, C., Sehgal, R., Ibanez, C.F. and Kogner, P. (1997) Coexpression of mRNA for the full-length neurotrophin receptor trk-C and trk-A in favourable neuroblastoma. *Eur J Cancer*, **33**, 2058-2063.

Takita, J., Yang, H.W., Chen, Y.Y., Hanada, R., Yamamoto, K., Teitz, T., Kidd, V. and Hayashi, Y. (2001) Allelic imbalance on chromosome 2q and alterations of the caspase 8 gene in neuroblastoma. *Oncogene*, **20**, 4424-4432.

Teitelman, G., Joh, T.H., Park, D., Brodsky, M., New, M. and Reis, D.J. (1982) Expression of the adrenergic phenotype in cultured fetal adrenal medullary cells: role of intrinsic and extrinsic factors. *Dev Biol*, **89**, 450-459.

Teitz, T., Wei, T., Liu, D., Valentine, V., Valentine, M., Grenet, J., Lahti, J.M. and Kidd, V.J. (2002) Caspase-9 and Apaf-1 are expressed and functionally active in human neuroblastoma tumor cell lines with 1p36 LOH and amplified MYCN. *Oncogene*, **21**, 1848-1858.

Thornberry, N.A. and Lazebnik, Y. (1998) Caspases: enemies within. *Science*, **281**, 1312-1316.

Tweddle, D.A., Malcolm, A.J., Bown, N., Pearson, A.D. and Lunec, J. (2001) Evidence for the development of p53 mutations after cytotoxic therapy in a neuroblastoma cell line. *Cancer Res*, **61**, 8-13.

Vaillant, A.R., Mazzoni, I., Tudan, C., Boudreau, M., Kaplan, D.R. and Miller, F.D. (1999) Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. *J Cell Biol*, **146**, 955-966.

Verdi, J.M. and Anderson, D.J. (1994) Neurotrophins regulate sequential changes in neurotrophin receptor expression by sympathetic neuroblasts. *Neuron*, **13**, 1359-1372.

Vivanco, I. and Sawyers, C.L. (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*, **2**, 489-501.

Vogan, K., Bernstein, M., Leclerc, J.M., Brisson, L., Brossard, J., Brodeur, G.M., Pelletier, J. and Gros, P. (1993) Absence of p53 gene mutations in primary neuroblastomas. *Cancer Res*, **53**, 5269-5273.

Walsh, G.S., Krol, K.M. and Kawaja, M.D. (1999) Absence of the p75 neurotrophin receptor alters the pattern of sympathosensory sprouting in the trigeminal ganglia of mice overexpressing nerve growth factor. *J Neurosci*, **19**, 258-273.

Wartiovaara, K., Barnabe-Heider, F., Miller, F.D. and Kaplan, D.R. (2002) N-myc promotes survival and induces S-phase entry of postmitotic sympathetic neurons. *J Neurosci*, **22**, 815-824.

Weiss, W.A., Aldape, K., Mohapatra, G., Feuerstein, B.G. and Bishop, J.M. (1997) Targeted expression of MYCN causes neuroblastoma in transgenic mice. *Embo J*, **16**, 2985-2995.

White, P.S., Maris, J.M., Beltinger, C., Sulman, E., Marshall, H.N., Fujimori, M., Kaufman, B.A., Biegel, J.A., Allen, C., Hilliard, C. and et al. (1995) A region of consistent deletion in neuroblastoma maps within human chromosome 1p36.2-36.3. *Proc Natl Acad Sci U S A*, **92**, 5520-5524.

White, P.S., Thompson, P.M., Seifried, B.A., Sulman, E.P., Jensen, S.J., Guo, C., Maris, J.M., Hogarty, M.D., Allen, C., Biegel, J.A., Matise, T.C., Gregory, S.G., Reynolds, C.P. and Brodeur, G.M. (2001) Detailed molecular analysis of 1p36 in neuroblastoma. *Med Pediatr Oncol*, **36**, 37-41.

Whitfield, J., Neame, S.J., Paquet, L., Bernard, O. and Ham, J. (2001) Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. *Neuron*, **29**, 629-643.

Woessmann, W., Chen, X. and Borkhardt, A. (2002) Ras-mediated activation of ERK by cisplatin induces cell death independently of p53 in osteosarcoma and neuroblastoma cell lines. *Cancer Chemother Pharmacol*, **50**, 397-404.

Wu, X. and Deng, Y. (2002) Bax and BH3-domain-only proteins in p53-mediated apoptosis. *Front Biosci*, **7**, d151-156.

Wu, Y., Mehew, J.W., Heckman, C.A., Arcinas, M. and Boxer, L.M. (2001) Negative regulation of bcl-2 expression by p53 in hematopoietic cells. *Oncogene*, **20**, 240-251.

Xu, Z., Maroney, A.C., Dobrzanski, P., Kukekov, N.V. and Greene, L.A. (2001) The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. *Mol Cell Biol*, **21**, 4713-4724.

Yamashiro, D.J., Liu, X.G., Lee, C.P., Nakagawara, A., Ikegaki, N., McGregor, L.M., Baylin, S.B. and Brodeur, G.M. (1997) Expression and function of Trk-C in favourable human neuroblastomas. *Eur J Cancer*, **33**, 2054-2057.

Yamashiro, D.J., Nakagawara, A., Ikegaki, N., Liu, X.G. and Brodeur, G.M. (1996) Expression of TrkC in favorable human neuroblastomas. *Oncogene*, **12**, 37-41.

Yan, C., Liang, Y., Nylander, K.D. and Schor, N.F. (2002) TrkA as a life and death receptor: receptor dose as a mediator of function. *Cancer Res*, **62**, 4867-4875.

Yuste, V.J., Sanchez-Lopez, I., Sole, C., Encinas, M., Bayascas, J.R., Boix, J. and Comella, J.X. (2002) The prevention of the staurosporine-induced apoptosis by Bcl-X(L), but not by Bcl-2 or caspase inhibitors, allows the extensive differentiation of human neuroblastoma cells. *J Neurochem*, **80**, 126-139.

Zhao, R., Gish, K., Murphy, M., Yin, Y., Notterman, D., Hoffman, W.H., Tom, E., Mack, D.H. and Levine, A.J. (2000) Analysis of p53-regulated gene expression patterns using oligonucleotide arrays. *Genes Dev*, **14**, 981-993.

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Appendix I: Research Compliance Certificates

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
David Kaplan	BTRC	x				
Christine Laliberte	BTRC		x			
Nick Marsh	BTRC					x
Jean-Francois Lavoie	BTRC				x	
Catherine Dubreuil	BTRC				x	
Mathieu Boudreau	BTRC				x	
Frederic Sweeney	BTRC			x		
Alexandre Angers-Loustau	BTRC				x	
Steeve Radinovic	BTRC				x	
Jane Ng	BTRC			x		
Gabrielle Molenkamp	BTRC					x
Kirmo Wartiovaara	BTRC					x
Jasi Atwal	BTRC				x	
Etienne Rousselle	BTRC				x	
Donna Senger	BTRC					x
Doron Lederfein	BTRC					x
Karl Fernandes	BTRC					x
Ute Zirrgiebel	BTRC					x

5. EMERGENCY: Person(s) designated to handle emergencies

Name: Dr David Kaplan Phone No: work: (514)398-2634 home: (514) 482-2186

Name: Dr Nick Marsh Phone No: work: (514)398-8436 home: (514) 948-2422

Briefly describe:

i) the biohazardous material involved & designated biosafety risk group

The material involved is the virus *adenovirus*: the risk group is #2.

ii) the procedures to be conducted

Our main use of adenovirus is in controlled tissue culture/cell culture experiments. The virus is used as a vector to insert certain specific (non-hazardous) genetic material of a primarily neural origin (such as cells from the cortex). The virus is attenuated, and the genetic material they carry may cause a phenotypic effect but is not contagious. All work is done in Class II laminar flow hoods.

iii) the protocol for decontaminating spills

For a small spill, the area is covered with, and wiped with, paper towel that has been soaked in bleach (1% bleach, for at least 10 minutes). The work area is wiped down before and after work with 70% ethanol. If a large spill (>500 mls occurs), then it is allowed to settle for 30 minutes, before being decontaminated as above. If the virus gets on skin, then that area is disinfected at once (medical help sought if necessary). Any contaminated clothing or materials are autoclaved. A separate incubator is used to grow the cells so they do not contact other cells, and this incubator is cleaned on a regular basis (70% ethanol and autoclaving).

Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent (s)?

NO.

Do procedures involving genetically engineered organisms have a history of safe use?

YES.

What precautions are being taken to reduce production of infectious droplets and aerosols?

e tissue culture work will be performed in Class II laminar flow floods. Whenever work is done, there will ays be an autoclavable beaker of !% bleach at hand, to decontaminate small items (eg: pipette tips, pasteur ettes); and spray containers of 1% bleach and of 70% ethanol are in close proximity to the work. Personnel king with the virus will wear lab coats, and gloves (which are frequently changed).
vacuum line for the flow hood has a double trap and is connected to a HEPA filter to ensure that no virus pes into the line. All the virus waste is autoclaved prior to disposal. The tissue culture room is separate from general labs.

10. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
BTRC	BT-102	Forma Scientific	1286	17719-312	11/29/1999
BTRC	BT-102	Forma Scientific	1286	17719-311	11/29/1999
BTRC	BT-102	Forma Scientific	1286	19727-301	11/29/1999